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APPLICANT(S) : MOSES RODRIGUEZ ET AL.
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FILED : August 8, 1996
FOR : PROMOTION OF CENTRAL NERVOUS SYSTEM
REMYELINATION USING MONOCLONAL
AUTOANTIBODIES

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STATEMENT IN SUPPORT OF THE FILING/SUBMISSION OF A
NUCLEOTIDE/AMINO ACID SEQUENCE LISTING IN
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08/692084

Case Docket No.: 1199-1-001 CIP

PATENT APPLICATION TRANSMITTAL LETTER

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Sir:

Transmitted herewith for filing is the patent application of:

Inventor(s) : MOSES RODRIGUEZ ET AL.For : PROMOTION OF CENTRAL NERVOUS SYSTEM
REMYELINATION USING MONOCLONAL AUTOANTIBODIES

Enclosed are:

- ☒ 18 sheets of informal drawings.
- ☐ An Assignment of the invention, to:
- ☐ An Information Disclosure Statement, Form PTO 1449 and cited references.
- ☐ A Verified Statement to establish Small Entity status under 37 C.F.R. 1.9 and 37 C.F.R. 1.27.
- ☐ Executed ☒ unexecuted Declaration and Power of Attorney.
- ☒ A Filing Date as of the date of deposit in Express Mail is requested. A Certificate of Mailing under 37 C.F.R. 1.10 is presented herewith.

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Respectfully submitted,


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several naturally occurring viral infections, both in humans and animals, can cause demyelination. One commonly utilized experimental viral model is induced by Theiler's murine encephalomyelitis virus (TMEV) (Dal Canto, M.C., and Lipton, H.L., Am. J. Path., 88:497-500 (1977)).

The limited efficacy of current therapies for MS and other demyelinating diseases, has stimulated interest in novel therapies to ameliorate these diseases. However, due to the apparently complex etiopathogenesis of these diseases, potentially involving both environmental and autoimmune factors, the need still exists for an effective treatment of these demyelinating disorders.

SUMMARY OF THE INVENTION

The present invention relates to the promotion, or stimulation, or remyelination of central nervous system axons in a mammal. Specifically, the present invention relates to methods of stimulating the remyelination of central nervous system (CNS) axons using autoantibodies of the IgM subtype, or active fragments thereof, characterized by their polyreactivity, and encoded by unmutated germline genes. These monoclonals (mAbs) are referred to herein as SCH94.03, SCH 79.08, O1, O4, A2B5 and HNK-1. Of these monoclonal antibodies, O1, O4, A2B5 and HNK-1 are well-known oligodendrocyte-reactive (OL-reactive) monoclonal antibodies. See, for instance, Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76 (1979), 4913-4917, and Abo et al. J. Immunol., 127 (1981), 1024-1029). The monoclonal antibodies referred to as SCH94.03 and SCH 79.08, and the corresponding hybridomas producing them, have been deposited on April 28, 1994, and February 27, 1996, respectively, under the terms of the Budapest Treaty, with the American Type Culture Collection (ATCC)

and given ATCC Accession Nos. CRL 11627 and HB12057, respectively.

The present invention utilizes an analysis of the Ig variable region cDNA sequences and the polyreactivity of these mAbs by ELISA to ascertain their utility in the methods described herein. Further, this work provides confirmation of the generic utility of this group of germline natural autoantibodies as effective in producing remyelination of the central nervous system.

10 The present invention also relates to methods of treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases of the central nervous system of humans and domestic animals, such as post-infectious encephalomyelitis, or prophylactically
15 inhibiting the initiation or progression of demyelination in these disease states, using the monoclonal antibodies, or active fragments thereof, of this invention. This invention further relates to *in vitro* methods of producing, and stimulating the proliferation of, glial
20 cells, such as oligodendrocytes, and the use of these glial cells to treat demyelinating diseases.

It is thus an object of the present invention to provide methods for treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases
25 of the central nervous system of humans and domestic animals, such as post-infectious encephalomyelitis, or prophylactically inhibiting the initiation or progression of demyelination in these disease states, using the described monoclonal autoantibodies, active fragments
30 thereof, or other natural or synthetic autoantibodies having the characteristics of mAb SCH94.03, SCH 79.08, 01, 04, A2B5 and HNK-1.

It is further an object of the present invention to provide *in vitro* methods of producing, and stimulating the proliferation of, glial cells, such as oligodendrocytes, and the use of these glial cells to
 5 treat demyelinating diseases.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1 is a graph depicting the dose-response characteristics of antibody-mediated proliferation of cells in mixed rat brain culture.

- 10 Figure 2 is a graph depicting the temporal profile of antibody-mediated proliferation of cells in mixed rat brain culture.

Figure 3A-3D shows light and electron micrographs of CNS remyelination promoted by mAb SCH94.03. (A) Light
 15 micrograph of spinal cord section from a chronically infected SJL/J mouse treated with SCH94.03 showing CNS remyelination. (B) Light micrograph of spinal cord section from a chronically infected SJL/J mouse treated with a control IgM showing extensive demyelination, and
 20 the relative absence of remyelination. Inflammatory cells, including macrophages with ingested myelin debris are indicated by arrows. The asterisk indicates a representative naked axon. (C) Light micrograph of spinal cord section with normal myelin. (D) Electron
 25 micrograph of spinal cord section from an animal treated with SCH94.03 showing multiple axons with abnormally thin myelin sheaths relative to axon diameter. The star in the upper right-hand corner indicates an axon with normal myelin sheath thickness. Arrowheads point to astrocytic
 30 processes, which are intimately associated with remyelinated axons. Scale bars represent 13 μm in A-C, and 2 μm in D.

Figure 4 is a graph depicting the correlation between the change in clinical disease and morphological remyelination.

Figure 5 is a graph depicting the dose-response relationship between treatment with mAb SCH94.03 and CNS remyelination. Area of CNA remyelination (•) and percentage of lesion area with remyelination (o) in animals treated with various doses of mAb SCH94.03.

Figure 6 shows a Western blot of TMEV proteins. Lysates from infected L2 fibroblast cells were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with SCH94.03 (lane 1), SCH93.32 (lane 2), serum from susceptible mice chronically infected with TMEV (lane 3), and polyclonal rabbit anti-TMEV IgG (lane 4). Molecular weights are indicated on the left in kilodaltons (kDa). The position and identification of the major TMEV capsid proteins are indicated on the right.

Figure 7A-7D shows the immunostaining of cultured glial cells and frozen CNS tissue sections with mAb SCH94.03. Scale bars represent 15 μ m.

Figure 8A-8C shows the results of SCH94.03 (Figure 8A) and control IgMs (Figure 8B and 8C) binding to protein antigens as determined by ELISA.

Figure 9 shows the results of SCH94.03 F(ab2)' binding to protein antigens as determined by ELISA.

Figure 10A-10C show the results of SCH94.03 (Figure 10A) and control IgMs (Figure 10B and 10C) binding to chemical haptens as determined by ELISA.

Figure 11 shows the alignment of the immunoglobulin light and heavy chain variable region sequences of SCH94.03 and control IgM, CH12, and germline Ig gene segments.

Figure 12 shows the nucleotide and deduced amino acid sequences of V_H , D and J_H regions encoding O1, compared with the unrearranged V_H segment transcript A1 and A4, and the J_H germline gene (SEQ. ID 1). Dashed lines indicate identity with unrearranged V_H segment transcript A1 and A4. Underline indicates identity with germline AP2 gene family (DSP2.3, 2.4, 2.6). Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession number L41877.

Figure 13 shows the nucleotide and deduced amino acid sequences of V_H , D and J_H regions encoding O4 and HNK-1 (SEQ. ID 2), compared with those reported for germline gene V_H 101 and J_H , and for natural autoantibody D23. Dashed lines indicate identity with V_H 101 and J_H 4. Underline indicates identity with germline DFL16.1. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. These sequences have been assigned the GenBank TM/EMBL Data Bank Accession Numbers L41878 (O4) and L41876 (HNK-a).

Figure 14 shows the nucleotide and deduced amino acid sequences of V_H , D and J_H regions encoding A2B5 (SEQ. ID 3), compared with those reported for germline gene V1 and J_H 3 germline gene. Dashed lines indicate identity with germline gene V1 and J_H 3. Underline indicates identity with germline DFL16.2. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has

been assigned the GenBank TM/EMBL Data Bank Accession Number L41874.

Figure 15 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding O1 and O4 (SEQ. ID 5 4), compared with those reported for myeloma MOPC21, for natural autoantibody E7 and for 3_x2 germline gene. Dashed lines indicate identity with MOPC21 and germline gene J_H2 (N, undetermined nucleotide). Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. These sequence have been assigned the GenBank TM/EMBL Data Bank Accession Numbers L41879 (O1) and L41881 (O4).

Figure 16 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding HNK-1 (SEQ.ID 5), compared with those reported for germline V_H41, myeloma MOPC21, and J_H2. Dashed lines indicate identity with germline genes. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession Number L41880.

Figure 17 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding A2B5 (SEQ ID 6). Dashed lines indicate identity with germline J_H. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession Number L41875.

Figure 18 is a graph showing the reactivity of O1, O4, A2B5 and control (TEPC183 and XXMEN-OES) IgM \times mAbs by direct ELISA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the promotion, or stimulation, or remyelination of central nervous system axons in a mammal. Specifically, the present invention
5 relates to methods of stimulating the remyelination of central nervous system (CNS) axons using a monoclonal autoantibody of the IgM subtype, or an active fragment thereof, characterized by its polyreactivity and encoded by unmutated germline genes, or a natural or synthetic
10 analog thereof.

As used herein, the term "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term is intended to encompass polyclonal, monoclonal, and
15 chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. Such antibodies include both polyclonal and monoclonal antibodies prepared by known generic techniques, as well as bi-specific (chimeric) antibodies,
20 and antibodies including other functionalities suiting them for additional diagnostic use conjunctive with their capability of modulating ~ activity stimulating the remyelination of CNS axons. An "antibody combining site" is that structural portion of an antibody molecule
25 comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen. The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active
30 portion of an immunoglobulin molecule. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the

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paratope, including those portions known in the art as Fab, Fab', F(ab'), and F(v).

Fab and F(ab')₂ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chains portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

15 The phrase "monoclonal antibody" in its various
grammatical forms refers to an antibody having only one
species of antibody combining site capable of
immunoreacting with a particular antigen. A monoclonal
antibody thus typically displays a single binding
20 affinity for any antigen with which it immunoreacts. A
monoclonal antibody may therefore contain an antibody
molecule having a plurality of antibody combining sites,
each immunospecific for a different antigen; e.g., a
bispecific (chimeric) monoclonal antibody.

25 The present invention also relates to methods of treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases of the central nervous system of humans and domestic animals, such as

30 post-infectious encephalomyelitis, using the SCH 94.03, SCH 79.08, O1, O4, A2B5 and HNK-1 monoclonal antibodies, an active fragment thereof, or a natural or synthetic autoantibody having the characteristics thereof. Methods of prophylactic treatment using these mAb, active

35 fragments thereof, or other natural or synthetic

autoantibodies having the same characteristics, to inhibit the initiation or progression demyelinating diseases are also encompassed by this invention.

Oligodendrocytes (OLs), the myelin-forming cells of the central nervous system (CNS), originate as neuroectodermal cells of the subventricular zones, and then migrate and mature to produce myelin. The sequential development of OLs is identified by well-characterized differentiation stage-specific markers.

Proliferative and migratory bipolar precursors, designated oligodendrocyte/type-3 astrocyte (O-2A) progenitors, are identified by monoclonal antibodies (mAbs) anti-GD₃ and A2B5 [Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76 (1979), 4913-4917]. The next developmental stage, characterized by multipolar, postmigratory, and proliferative cells, is recognized by mAb O4 [Gard et al., Neuron, 5 (1990), 615-625; Sommer et al., Dev. Biol., 83 (1981), 311-327]. Further development is defined by the cell surface expression of galactocerebroside, recognized by mAb O1 [Schachner, J. Neurochem., 39 (1982), 1-8; Sommer et al., *supra*], and by the expression of 2',3'-cyclic nucleotide 3'-phosphohydrolase. The most mature cells express terminal differentiation markers such as myelin basic protein and proteolipid protein.

The mAbs (A2B5, O1, and O4) used to characterize the stages of OL development were made by immunizing BALB/c mice with chicken embryo retina cells or homogenate of bovine corpus callosum [Eisenbarth et al., *supra*; Sommer et al., *supra*]. A2B5 recognizes not only O-2A progenitors but also neurons and reacts with cell surface ganglioside GQ1c [Kasai et al., Brain Res., 277 (1983), 155-158] and other gangliosides [Fredman et al., Arch. Biochem. Biophys., 233 (1984), 661-666]. O4 reacts with sulfatide, seminolipid and cholesterol [Bansal et al.,

J. Neurosci. Res., 24 (1989), 548-557], whereas O1 reacts with galactocerebroside, monogalactosyl-diglyceride and psychosine [Bansal et al., *supra*]. These mAbs belong to the IgM immunoglobulin (Ig) subclass and recognize

5 cytoplasmic structures as well as the surface antigens of OLs [Eisenbarth et al., *supra*; Sommer et al., *supra*]. Mouse mAb HNK-1 (anti-Leu-7), made by immunizing BALB/c mice with the membrane suspension of HSB-2 T

10 lymphoblastoid cells, was first reported as a marker for natural killer cells [Abo et al., J. Immunol., 127 (1981), 1024-1029]. Later, HNK-1 was shown to share antigenic determinants with the nervous system [Schuller-Petrovic et al., Nature, 306 (1983), 179-181]. The

15 carbohydrate epitope on myelin-associated glycoprotein, found in both central and peripheral myelin sheaths, was shown to be a principal antigen of nervous tissue the reacted with HNK-1 [McGarry et al., Nature, 306 (1983), 376-378]. However, other glycoproteins in nervous tissue react with this mAb, some of which are important in

20 embryogenesis, differentiation, and myelination [Keilhauer et al., Nature, 316 (1985), 728-730; Kruse et al., Nature, 311 (1984), 153-155; Kruse et al., Nature, 316 (1985), 146-148; McGarry et al., J. Neuroimmunol., 10 (1985), 101-114]. Of interest, HNK-1 also reacts with

25 cytoplasmic structures and belongs to the IgM Ig subclass.

A monoclonal antibody, disclosed and claimed in copending parent application U. S. S. N. 08/236,520, filed April 29, 1994, and designated SCH94.03, was found to promotes

30 CNS remyelination in mice infected chronically with Theiler's murine encephalomyelitis virus (TMEV) [Miller et al., J. Neurosci., 14 (1994), 6230-6238]. SCH94.03 belongs to the IgM(x) Ig subclass and recognizes an unknown surface antigen on OLs, but cytoplasmic antigens

35 in all cells (Asakura et al., Molecular Brain Research, in press). The polyreactivity of SCH94.03 by ELISA, and

the unmutated Ig variable region germline sequences indicated that SCH94.03 is a natural autoantibody [Miller et al., J. Neurosci., 14 (1994), 6230-6238]. A close study of SCH94.03, and comparison thereof with well-known

5 OL-reactive mAbs A2B5, O1, O4, and HNK-1 raised the possibility that these are natural autoantibodies. A subsequent analysis of the Ig variable region cDNA sequences and the polyreactivity of these mAbs by ELISA confirmed that this is a generic group of natural

10 autoantibodies having similar utilities.

The antigen reactivity of the monoclonal antibody, IgM monoclonal antibody referred to herein as SCH 94.03 (also referred to herein as SCH94.32) and SCH 79.08 (both prepared from a mammal immunized with spinal cord

15 homogenate from a normal mammal (i.e., uninfected with any demyelinating disease)), have been characterized and described in the aforesaid parent Application U.S.S.N. 08/236,520, filed April 29, 1994, whose teachings are incorporated herein by reference, using several

20 biochemical and molecular assays, including immunohistochemistry, immunocytochemistry, Western blotting, solid-phase enzyme-linked immunosorbant assays (ELISA), and Ig variable region sequencing. The hybridomas producing monoclonal antibody SCH 94.03 and

25 SCH 79.08 have been deposited on April 28, 1994, and February 27, 1996, respectively, under the terms of the Budapest Treaty, with the American Type Culture Collection (ATCC) and have been given ATCC Accession Nos. CRL 11627 and HB12057, respectively.

30 All restrictions upon the availability of the deposit material will be irrevocably removed upon granting of a patent.

Natural or physiologic autoantibodies are present normally in serum, are polyreactive, are frequently of

35 the IgM subtype, and are encoded by unmutated germline

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genes. By sequencing immunoglobulin (Ig) cDNAs of the oligodendrocyte-reactive O1, O4, A2B5, and HNK-1 IgM \times monoclonal antibodies and comparing these with published germline sequences, it was determined that these were

5 natural autoantibodies. O1 V_H was identical with unrearranged V_H segment transcript A1 and A4, O4 V_H had three and HNK-1 V_H had six nucleotide differences from germline V_H101 in the V_H coding region. The D segment of O1 was derived from germline SP2 gene family, J_H4,

10 whereas O1 J_H was encoded by germline J_H1 with one silent nucleotide change. O1 and O4 light chains were identical with myeloma MOPC21 except for one silent nucleotide change. HNK-1 V _{κ} was identical with germline V _{κ} 41 except for two silent nucleotide changes. O1 J _{κ} , O4J _{κ} and HNK

15 J _{κ} were encoded by unmutated germline J _{κ} 2. In contrast, A2B5 V_H showed seven nucleotide differences from germline V1, whereas no germline sequence encoding A2B5 V _{κ} was identified. O1 and O4, but not A2B5 were polyreactive against multiple antigens by direct ELISA. Therefore,

20 O1, O4 and HNK-1 Igs are encoded by germline genes, and have the genotype and phenotype of natural autoantibodies.

Selection of SCH mAbs to promote CNS remyelination

A panel of monoclonal antibodies (mAbs) derived from

25 splenocytes of uninfected SJL/J mice injected with SCH was constructed as described in detail in Example 1. After the initial fusion and cloning, 2 of the 95 wells with viable Ig-secreting hybridomas contained mAb with significant binding to SCH as demonstrated by ELISA.

30 Hybridoma cells from these two wells, called the 79 and 94 series, were subcloned by limiting dilution and screened again for binding to SCH by ELISA. For the 79 series hybridomas, 14 out of 49 clones were positive by SCH ELISA, while for the 94 series, 17 out of 32 were

35 positive for binding to SCH. Based upon the ELISA data,

two 79 series hybridomas (SCH79.08 and SCH79.27), both of which also reacted with myelin basic protein (MBP) by ELISA, and three 94 series hybridomas (SCH94.03, SCH94.11, and SCH94.32), none of which reacted with MBP, were chosen for ascites production and *in vivo* transfer experiments.

mAbs Promote Proliferation of Glial Cells

As described in Example 2, the mAbs were tested for their ability to promote proliferation of glial cells *in vitro*.

The dose-response characteristic of antibody-mediated proliferation were then examined. As shown in Figure 1, maximal stimulation with 94.03 was seen at 100 ng/ml. Control myeloma IgMs MOPC 104E and TEPC 183 (data not shown) also stimulated the mixed rat brain cultures to proliferate. However, the maximal effect was seen at a 10-fold higher concentration than that seen with the mAbs.

The temporal profile of antibody-mediated proliferation was also examined as shown in Figure 2. On day 8, after culture initiation, 100 ng/ml antibody was added to the cultures (time 0). Cells were harvested at 24 hour intervals; [³H]thymidine was present for the final 24 hours of culture to measure the total proliferation during the interval. The maximal stimulation with 94.03 was seen at 72 hours after antibody addition. Similar results were obtained with 94.32. None of the isotype control antibodies showed any significant proliferation throughout the 120 hours of culture. These data demonstrates that both mAbs 94.32 and 94.03 induce proliferation of glial cells of mixed rat brain culture. This proliferation is maximal at an antibody concentration of 100 ng/ml and a culture period of 72 hours after antibody addition.

CNS Remyelination Promoted by mAbs SCH94.033 and SCH94.32

- As described in Example 3, SJL/J mice chronically infected with TMEV were treated with a total mAb dose of 0.5 mg iv or 5.0 mg ip divided into twice weekly doses
- 5 for 4-5 weeks. CNS remyelination was measured by a quantitative morphological assessment on ten spinal cord cross-sections from each mouse. The criterion for CNS remyelination was abnormally thin myelin sheaths relative to axonal diameter. The data are composite of six
- 10 experiments and are presented as the \pm SEM, where n indicates the number of mice. Statistical comparisons for remyelination data were made with the cumulative values from both IgM and buffer only controls using a modified rank sum test. The number of demyelinated
- 15 lesions and the area of demyelination were not significantly different between treatment groups assessed by a one-way ANOVA. For control IgMs, myelomas MOPC 104E and ABPC 22 (both from Sigma), and TB5-1, an anti-mycobacteria mAb, were used.
- 20 SJL/J mice chronically infected with TMEV and treated with either mAb SCH94.03 or SCH94.32 showed significantly greater CNS remyelination than animals treated with either isotype-matched control mAb or buffer only (Table 1).

TABLE 1.

Monoclonal antibodies SCH94.03 and SCH94.32 promote CNS remyelination							
Treatment	n	Number of Demyelination (lesions)	Number of Remyelinated Lesions	p-value	Area of Remyelination (mm ²)	Area of Lesion (mm ²)	Area Remyelination /area Lesion (%)
SCH94.03	12	25.6 ± 2.6	12.8 ± 2.6	<0.0025	0.35 ± 0.09	1.09 ± 0.19	28.9 ± 3.8
SCH94.32	12	24.9 P ± 2.8	12.3 ± 2.3	<0.0001	0.42 ± 0.11	1.46 ± 0.21	26.7 ± 4.2
IgM control	13	29.9 ± 2.0	6.7 ± 1.2	--	0.11 ± 0.02	1.70 ± 0.28	7.7 ± 1.8
Buffer only	11	27.7 ± 2.7	5.1 ± 1.1	--	0.06 ± 0.01	1.11 ± 0.29	6.5 ± 1.2

Remyelination was seen with either iv or ip injections. SCH94.03- or SCH94.32-treated animals had approximately 2-3- fold more remyelinated lesions, and a 3-4-fold larger total area of CNS remyelination than control animals. When a cumulative statistical comparison was made using these two parameters of therapeutic effectiveness, the CNS remyelination induced by mAbs SCH94.03 and SCH94.32 was highly significant ($p < 0.005$; Table 2). In a chronic progressive disease like TMEV infection, the extent of CNS repair is a direct function of the extent of CNS damage. Both the number and area of CNS lesions were not different between treatment groups, indicating similar disease severity (Table 1). When CNS remyelination was expressed as the percentage of lesion area showing remyelination, approximately one-third of the cumulative demyelinated lesion area shown CNS remyelination in mice treated with either mAb SCH94.03 or SCH94.32 (Table 1).

Similar results were obtained using Schh 79.08 (Results shown in Table 2) and for O1, O4, A2B5 and HNK-1 (Results shown in Table 3).

Table 2. Enhancement of CNS remyelination by SCH79.08

Treatment	No. of Mice	Area of white matter (mm ²)	Area of CNS-type remyelination (mm ²)	Area of demyelinated lesion (mm ²)	Area of CNS-type remyelination/area of lesions (%)
SCH79.08	15	8.42 ± 0.33	0.20 ± 0.05	1.01 ± 0.16	20.2 ± 4.7
PBS	6	8.89 ± 0.26	0.03 ± 0.01	1.01 ± 0.21	2.4 ± 0.8

5 Values represent the mean ± SEM.

Statistics byf student t-test comparing area of CNS-type remyelination/area of lesions revealed $p < 0.05$.

PBS: phosphate buffered saline.

Table 3. Enhancement of CNS remyelination by oligodendrocyte-reactive monoclonal antibodies

Treatment	No. of mice	Area of White matter (mm ²)	Area of CNS-type remyelination (mm ²)	Area of demyelinated lesion (mm ²)	Area of CNS-type remyelination/area of lesions (%)
O1	6	7.57 ± 0.52	0.14 ± 0.04	0.53 ± 0.10	24.8 ± 6.2*
O4	7	8.01 ± 0.15	0.17 ± 0.04	0.84 ± 0.10	20.4 ± 4.2*
A2B5	7	7.28 ± 0.38	0.18 ± 0.05	0.70 ± 0.17	24.6 ± 4.6*
HNK-1	7	7.16 ± 0.38	0.15 ± 0.03	0.78 ± 0.10	20.6 ± 2.8*
PBS	6	7.46 ± 0.70	0.05 ± 0.02	0.51 ± 0.14	8.0 ± 2.2

Values represent the mean ± SEM.

10 Statistics by student t-test comparing area of CNS-type remyelination/area of lesions revealed *:p<0.05, **:p<0.01.

PBS: phosphate buffered saline

Morphology of CNS Remyelination

CNS remyelination was readily identified morphologically both by light and electron microscopy (Figure 3A-3D). Figure 3A shows a remyelinated lesion from an animal
 5 treated with SCH94.03. The majority of axons in the lesion show morphologic evidence of repair, with abnormally thin myelin sheaths relative to axonal diameter (Ludwin, S.K. "Remyelination in the central nervous system of the mouse," In: THE PATHOLOGY OF THE
 10 MYELINATED AXON (Adachi M, Hirano A, Aronson SM eds), pp 49-79, Tokyo: Igaku-Shoin Ltd. (1985)). For comparison, Figure 3B shows a demyelinated lesion, with minimal remyelination, whereas Figure 3C is an area of normal myelin, with thickly myelinated axons. Within
 15 remyelinated lesions (Figure 3A), there were 15.3 ± 1.0 (mean \pm SEM) myelinated axons per $100 \mu\text{m}^2$, compared to only 1.1 ± 0.2 myelinated axons per $100 \mu\text{m}^2$ in demyelinated lesions (Figure 3B). Figure 3C shows a light micrograph of spinal cord section with normal
 20 myelin. By electron microscopy, CNS remyelination was especially evident (Figure 3D). Almost every axon in the field has evidence of new myelin formation, although the degree of remyelination (i.e., myelin thickness) is variable between individual axons, suggesting different
 25 stages of the repair process. The ratio of myelin thickness to axonal diameter was 0.08 ± 0.01 (mean \pm SEM; $n = 25$ axons) for remyelinated axons compared to 0.21 ± 0.01 ($n = 34$ axons) for normally myelinated axons.

Correlation Between Clinical Disease and Morphological

Remyelination

The correlation of morphological remyelination with clinical signs of disease improvement was assessed as described in Example 3. At each treatment injection, mice were assessed clinically as described in Example 3.
 35 The change in clinical score was correlated with the

percentage of lesion area showing remyelination (Figure 4). Morphological remyelination is represented as the percentage of lesion area showing CNS remyelination. A change in clinical score of 0 represent stable disease over the treatment period (4-5 weeks), whereas a positive change indicates worsening of clinical disease, and a negative change indicates improvement. Data represent individual animals from all treatment groups. A positive change in clinical score indicates worsening of disease.

Using data from all treatment groups, the change in clinical score showed a moderate but significant negative correlation ($R=-0.40$; $p < 0.04$) with the percentage of lesion area showing remyelination. Although few animals actually improved clinically (Δ clinical score < 0), animals with an increase in disease severity (Δ clinical score > 0) tended to have less morphological remyelination, while animals that remained stable clinically (Δ clinical score = 0) showed the most remyelination. A similar negative correlation was obtained when the other quantitative measures of remyelination were used (the number of remyelinated lesions and the area of remyelination) as shown in Table 1. These data demonstrate that remyelination quantitated by morphology is associated with slowing of clinical disease progression.

Titration of mAb SCH94.03 Dose and CNS Remyelination

For the initial treatment experiments, a total mAb dose of 25 mg/kg for intravenous (iv) injections and 250 mg/kg for intraperitoneal (ip) injection was empirically chosen. To assess the dose-response characteristics, and to determine the minimal amount of mAb needed to promote remyelination, chronically-infected mice were treated with various ip doses of SCH94.03. Remyelination was quantitated as described for Table 1. Data are the mean values of 4-5 animals per mAb dose, with the final cumulative dose indicated on the graph. SEM averaged 35%

of the mean. There was no statistical difference assessed by one-way ANOVA in the number of demyelinated lesions or the area of demyelination between treatment groups, indicated similar extent of disease in all
 5 animals. The number of demyelinated lesions and area of lesions were 33.2 ± 7.5 and 1.25 ± 0.43 for the 1000 μg group, 31.8 ± 8 and 1.11 ± 0.31 for the 100 μg group, 23.8 ± 3.4 and 0.54 ± 0.14 for the 10 μg group, and 20.0 ± 6.5 and 0.74 ± 0.20 for the buffer only group (represented
 10 as the 0 dose point on the graph). Animals treated with 100 μg control IgM (MOPC 104E) had remyelination scores similar to control animals treated with buffer only. The positive correlation between the dose of mAb SCH94.03 and CNS remyelination was especially striking when the
 15 severity of CNS disease was taken into account. When CNS repair was expressed as the percentage of lesion area showing remyelination, mice treated with a total dose of 1000, 100, or 10 μg of SCH94.03 had 6-, 5-, and 4-fold more remyelination than control animals, respectively
 20 (Figure 5). Mice given as little as 10 μg of SCH94.03 ip (0.5 mg/kg) showed evidence of enhanced CNS remyelination. These data indicated that mAb SCH94.03 and CNS remyelination had a positive dose-response relationship, and that very small quantities of mAb were
 25 needed to promote myelin repair.

Antigen Specificity of SCH94.03 and SCH94.32

Although mAbs SCH94.03 and SCH94.32 were generated from splenocytes of uninfected mice, and screened against SCH from uninfected mice, it was directly assessed whether
 30 either mAb could react with TMEV capsid proteins or inhibit viral infectivity *in vitro*. By Western blotting (Figure 6), SCH94.03 and SCH94.32 did not react with any TMEV proteins recognized by either serum from chronically infected mice or polyclonal IgG from rabbits injected
 35 with purified TMEV (Rodriguez, et al., Ann. Neurol., 13:426-433 (1983)). Western blot of lysates from control

mock infected L2 cells showed single bands with the serum from chronically infected animals and the polyclonal rabbit anti-TMEV IgG at 32 and 43 kDa, respectively, but no reactivity with SCH94.03 or SCH94.32.

- 5 In addition, no significant inhibition of TMEV infectivity *in vitro* with up to 5 μ g/ml of either SCH94.03 or SCH94.32, was observed under assay conditions where 50% neutralization was observed with a 1:34,000 dilution of serum from chronically infected animals.
- 10 These results indicated that the therapeutic effect of SCH94.03 and SCH94.32 was not due to direct inhibition of the virus.

To initially characterize the antigens recognized by mAbs SCH94.03 and SCH94.32, various cell lines derived from

- 15 glial (rat C6, mouse G26-20, human U373MG and U87MG), neural (human neuroblastoma), fibroblast (mouse L and 3T3), epithelial (human SCC-9 carcinoma), and lymphocytic (mouse CTLL2) origin were stained. Both mAbs stained internal antigens of all cell lines tested, which
- 20 indicated that certain antigens recognized by these mAbs were not restricted to unique cell types *in vitro*. Based on the hypothesis that the therapeutic effect of SCH94.03 and SCH94.32 was due to a CNS-specific interaction, the immunostaining of cultured cells by SCH94.03 and SCH94.32
- 25 using the rat glial cell line 5.5B8 was further investigated. This immortalized glial cell line has phenotypic characteristics of both oligodendrocytes and astrocytes, with expression of MBP and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), and low, but
- 30 detectable, expression of glial fibrillary acidic protein (GFAP) and the lipids or proteins recognized by the mAbs A2B5 and 04 (Bozyczko, et al., Ann. NY Acad. Sci., 605:350-353 (1990)). SCH94.03 and SCH94.32 recognized both a surface and cytoplasmic determinant on 5.5B8
- 35 cells. The surface staining was most prominent on small

cells which lay on top of a layer of flat, morphologically differentiated cells (Figure 7A). Surface staining was confirmed by flow cytometry on live cells. When the cell membrane was permeabilized by dehydration or brief treatment with a non-ionic detergent to expose internal antigens, the staining pattern was altered considerably (Figure 7B). The cytoplasmic staining was filamentous, with a dense perinuclear network that extended out into the cell processes. This pattern closely resembled the staining pattern of the intermediate filament cytoskeletal protein vimentin. These data indicated that SCH94.03 and SCH94.32 recognized antigens that were not restricted to cells derived from the nervous system, but that they did recognize both surface and cytoplasmic determinants on glial cells.

Immunohistochemical staining of frozen mouse, rat, and human tissue confirmed that SCH94.03 and SCH94.32 were not CNS-specific mAbs, but rather showed multi-organ reactivity. Both mAbs immunostained all major organs examined, including the brain, spinal cord, optic nerve, heart, liver, kidney, stomach, and small intestine and skeletal muscle. However, not all cells within an organ stained, suggesting *in situ* cytological specificity. Within the CNS, SCH94.03 and SCH94.32 stained predominately blood vessels, ependymal cells, and stellate-shaped cells with the morphological features of glial cells, which were enriched in neonatal cerebellar, periventricular, and brain stem white matter (Figure 7C), and both neonatal and adult optic nerve. Similar glial cells positive for SCH94.03 and SCH94.32 were found in autopsied human brain tissue, especially at the gray-white matter junction (Figure 7D). Identical immunostaining results were obtained with mAb SCH94.32. Immunostaining with a control IgM (MOPC 104E) was

negative for all samples and tissue structures which immunostained with SCH94.03 and SCH94.32.

The identification and characterization of an entire family of autoantibodies, referred to as "natural" or "physiological" autoantibodies, has influenced traditional view of autoimmunity and self-reactivity. The natural autoantibodies that have been studied extensively are typically IgMs, although other isotypes have been identified, are reactive toward a wide range of antigens, including cytoskeletal proteins, surface proteins, nucleic acids, phospholipids, bacterial antigens such as lipopolysaccharides, and various chemical haptens (reviewed by Avrameas and Ternynck, Mol. Immunol., 30:1133-1142 (1993)). Natural autoantibodies share extensive idiotypic cross-reactivity or "connectivity", which includes expression of similar idiotypes, some of which are expressed by pathogenic autoantibodies, as well as reactivity toward common idiotypes expressed on other antibodies. Molecular analysis has shown that natural autoantibodies are typically encoded by unmutated germline immunoglobulin (Ig) genes, with few if any somatic mutations, and therefore represent a substantial fraction of the Ig repertoire, especially in neonatal animals which have not had extensive exogenous antigen exposure.

The function of natural autoantibodies remains enigmatic. Several hypotheses have been proposed based upon their biochemical and molecular characteristics. These include: (1) clearance of senescent or damage tissue, (2) providing a first line of immunological defense in the lag period between pathogen exposure and an Ag-specific immune response, (3) masking autoantigens from a potentially pathogenic autoimmune response, (4) immunomodulation, including shaping of the neonatal immune repertoire via an idiotypic network, and (5)

participation in the positive selection of B cells in the bone marrow, similar to the process proposed for T cells in the thymus.

The hypothesis that antibodies SCH94.03 and SCH94.32 were natural autoantibodies was tested. To characterize the antigen reactivities of SCH94.03 and SCH94.32, several biochemical and molecular assays, including immunohistochemistry and immunocytochemistry, Western blotting, solid-phase enzyme-linked immunosorbant assays (ELISA), and Ig variable region sequencing, were used. As described below, for all biochemical assays, SCH94.03 and SCH94.32 were indistinguishable. In addition, SCH94.03 and SCH94.32 had identical Ig variable region sequences, which confirmed that they were the same mAb. Further details of these characterizing studies are reported in Asakura et al., J. Neuroscience Res. (1996) 43, pp 273-281, which disclosure is incorporated herein by reference.

A potential mechanism whereby SCH94.03 could stimulate remyelination in the central nervous system would be to stimulate the proliferation and/or differentiation of cells involved in myelinogenesis, primarily oligodendrocytes or their immature precursors. Thus, it was tested whether SCH94.03 stained the surface of various cells. Using immortalized cells, it was determined that SCH94.03 stained two glial cells lines, 5.5B8 (Figure 7A) and 20.2E11, but did not stain the surface of several other glial cells lines (10.1A3, 20.2A40, C6, G26-20), a neuroblastoma cell line (B104), two fibroblast lines (L2, Cos-1), or two myoblastomas (G8, L6). Similar results were obtained with cells isolated from animal tissues and grown in culture. SCH94.03 stained the surface of oligodendrocytes, but not astrocytes, microglia, Schwann cells, myoblasts, or fibroblasts.

The reactivity of SCH94.03 with proteins from glial and lymphoid cell lines, and tissue lysates from brain, liver, and intestine by Western blotting was also assessed. SCH94.03 reacted with multiple bands from all
 5 cells and tissues examined, with prominent reactivity towards bands at 50, 95, 120, and >200 kDa. The exact identity of these protein bands has not been determined.

The activity of SCH94.03 with several purified protein self-antigens by solid-phase ELISA was determined.
 10 (Figure 8A-8C). SCH94.03 showed strong reactivity toward the RBC antigen spectrin, but also showed consistent reactivity toward hemoglobin, actin, tublin, and vimentin, and thyroglobulin, although to a lesser qualitative degree than toward spectrin. No reactivity
 15 was observed with myosin, transferrin, albumin, lysozyme, or myelin basic protein under our assay conditions. Six other monoclonal or myeloma IgM controls XXMEN-OE5 (Figure 8B), A2B5, MOPC104E, TEPC183, 01, and CH12 (Figure 8C), were also tested, and no reactivity with any
 20 of the antigens tested was observed.

To confirm the monoclonality of SCH94.03, 18 subclones of SCH94.03 (9 each from SCH94.03 and SCH94.32 parents) were tested for polyreactivity by solid-phase ELISA. All 18 subclones showed identical reactivity patterns with the
 25 panel of protein antigens as the parent SCH94.03. To further support the conclusion that the polyreactivity of SCH94.03 was via its Fab region, we generated F(ab)₂' fragments and assessed their reactivity with the protein antigens by ELISA (Figure 9). SCH94.03 F(ab)₂' fragments
 30 showed similar polyreactivity as the whole IgM molecule.

A panel of chemical haptens coupled to bovine serum albumin (BSA) was constructed and used to assess SCH94.03 reactivity by solid-phase ELISA (Figure 10A-10C). SCH94.03 showed strong reactivity toward fluorescein (FL)

and 4-hydroxy-3-nitrophenyl acetic acid (NP), moderate reactivity toward phenyloxazolone (PhOx), and weak reactivity toward 2, 4, 6-trinitrophenyl (TNP) and *p*-azophenylarsonic acid (Ars). No reactivity with *p*-azophenyltrimethylammonium (TMA), *p*-azophenylphosphorylcholine (PC), or the carrier protein BSA was detected. Control IgMs (Figure 10B and 10C) showed no significant binding to any of the haptens tested, with the exceptions of CH12 reactivity with TMA, which has been previously reported, and A2B5 reactivity with NP.

It was further investigated whether the Ig light (L) and heavy (H) chains of SCH94.03 were encoded by germline Ig genes (Figure 11). The light chain variable (V_L) and joining (J_L) region nucleotide sequences from SCH94.03 had 99.4% identity with the previously published sequences of the germline $V_{\kappa 10}$ and $J_{\kappa 1}$ genes, with only two silent changes at the 3' end of both the V_L and J_L regions. The SCH94.03 V_H region nucleotide sequence was identical to the previously published germline V_{H23} sequence, the J_H region sequence differed from the published germline J_{H2} sequence by one nucleotide, at the 5' end of the J region, and the diversity (D) region contained 15 contiguous nucleotides derived from the germline DFL16.1 gene. There were 8 nucleotides in the V-D junction, and 1 in the D-J junction, which did not correspond to any known germline V or D region genes, and probably represent noncoded (N) nucleotides inserted by the enzyme terminal deoxynucleotide transferase during V-D-J recombination. The only changes from the germline genes in the heavy chain of SCH4.03 occurred at either the V-D or D-J junction, and therefore could represent either N nucleotides or the result of imprecise joining, rather than somatic mutations. In addition, both the light and heavy chain variable regions of SCH94.03 showed extensive

sequence similarity with the IgM produced by the B-cell lymphoma CH12 (Figure 11).

These antigen reactivity results suggest that SCH94.03 is a natural autoantibody. Although this conclusion does not readily present a mechanism as to how SCH94.03 stimulates remyelination in the central nervous system, it does suggest an important physiological function of natural autoantibodies. Autoantibodies that are produced either during normal physiology, or in response to tissue damage and the subsequent release of previously sequestered antigens, might actively participate to promote repair in the damaged tissue. In line with previously proposed functions of natural autoantibodies, this active participation might be to facilitate removal of damaged tissue, mask autoantigens thereby preventing vigorous pathogenic autoimmune response, modulate the immune response which actually resulted in the tissue destruction, thereby allowing normal endogenous tissue repair to occur, or directly stimulate cells involved in the repair process.

Thus, as a result of the work described herein, it is now demonstrated that an autoantibody generated and screened for its autoantigen-binding capability, also promotes CNS remyelination. Mice chronically infected with TMEV and treated either intravenously (iv) or intraperitoneally (ip) with IgM mAbs from hybridomas SCH94-03 or SCH94.32 had significantly more CNS repair than control animals, measured by a detailed quantitative morphological assessment of CNS remyelination. Moreover, preliminary data suggest that the autoantibody, SCH94.03 is also effective in preventing clinical relapses in mammals afflicted with experimental autoimmune encephalomyelitis (EAE).

Clinical disease in SJL/J mice with established R-EAE after treatment with SCH94.03. R-EAE was induced in SJL/J mice through adoptive transfer of MBP peptide (91-103)-specific T cells and treatment was initiated with

5 monoclonal autoantibody SCH94.03, control IgM, or PBS after recovery from the initial episode of clinical disease. Both the initial clinical disease peak and severity were similar between treatment groups (Table 4). However, treatment with SCH94.03 reduced the percentage

10 of mice with a first clinical relapse by half compared to mice treated with control IgM or PBS, and prolonged relapse onset by 6 days in those mice that did have a clinical relapse. When only mice with severe initial clinical disease (score ≥ 3) were analyzed, 10 of 12 mice

15 (83%) treated with control IgM or PBS had a first relapse compared to only 3 of 9 mice (33%) treated with SCH94.03 ($P < 0.04$ using a Fisher exact test), indicating that SCH94.03 was effective regardless of initial disease severity. In addition, 4 mice treated with control IgM

20 or PBS had a second clinical relapse, whereas no mouse treated with SCH94.03 had more than one relapse, although this difference was not statistically significant because of the few mice with a second relapse prior to sacrifice.

Spinal cord pathology in SJL/J mice with established R-EAE after treatment with SCH94.03. Treatment with

25 SCH94.03 also improved pathological disease in established R-EAE. Consistent with the reduction in clinical disease, treatment with SCH94.03d reduced by 40% both demyelination and meningeal inflammation in the

30 spinal cords of SJL/J mice with R-EAE (Table 5). Demyelinated lesions in mice treated with SCH94.03 were typically smaller in size with fewer inflammatory cells than mice treated with control IgM or PBS. The majority of demyelinated lesions were located in the dorsal

35 columns in mice treated with SCH94.03 ($57.0 \pm 5.4\%$; mean \pm SEM) and control IgM or PBS ($51.5 \pm 4.8\%$; $P > 0.4$ using

a Student's *t* test). The remainder of the demyelinated lesions in mice treated with SH94.03 or control IgM or PBS were distributed between posterolateral ($12.0 \pm 2.9\%$ and $11.0 \pm 2.0\%$, respectively), anterolateral ($14.3 \pm 3.0\%$ and $20.3 \pm 2.5\%$), and ventral ($14.9 \pm 17.1 \pm 1.6\%$) columns ($P > 0.1$ for all).

To evaluate the relationship between clinical and pathological disease in R-EAE, we correlated pathology scores (Table 5) with the severity of the initial clinical attack and any subsequent relapse (Table 4) in individual mice. Regression analyses indicated a moderate but statistically significant correlation between relapse severity and both demyelination ($r = 0.64$; $P > 0.6$). These results suggest that in addition to preventing demyelination and meningeal inflammation, the overall clinical benefits of SCH94.03 were secondary to inhibition of disease processes not readily identifiable by standard pathological analysis.

Table 4. Clinical Disease in SJL/L Mice With R-EAE After Treatment With SCH94.03

TREATMENT			
		SCH94.03	Control*
<u>Number of Mice</u>		14	19
5	<u>Initial attack</u>		
	Peak (day)	13 \pm 1 \ddagger	14 \pm 1
	Maximal clinical severity	2.8 \pm 0.2 \ddagger	2.8 \pm 0.2
	<u>First relapse</u>		
	No. mice relapsed (%)	5/14 (35.7) \S	15/19 (78.9)
10	Onset \P	2.4 \pm 2 **	18 \pm 2
	Maximal clinical severity	2.4 \pm 2 \ddagger	2.1 \pm 0.2
	<u>Second Relapse</u>		
	No. mice relapsed (%)	0/14 (0.0) $\ddagger\ddagger$	4/19 (21.1)
	Onset \P	-	29 \pm 2
15	Maximal clinical severity	-	2.3 \pm 0.4
	<u>Cumulative relapses</u>	4	19
	<u>Length of follow-up (days)</u>	56 \pm 1 \ddagger	58 \pm 1

SJL/J mice with R-EAE were injected with 50 μ g SCH94.03, IgM, or an equivalent volume of PBS twice weekly after spontaneous recovery from the initial episode of clinical disease. Subsequent relapses were assessed and graded

* Combined data from mice treated with control IgM ($n =$
5 10) or PBS ($n = 9$). No differences were observed with
any disease parameter between the two control groups.
‡ Not significant ($P > 0.05$) when compared to control
data using a Mann-Whitney rank sum test.
§ $P < 0.03$ when compared to control data using a Fisher
10 exact test.
¶ Number of days from the peak of the initial attack.
** $P < 0.05$ when compared to control data using a Mann-
Whitney rank sum test.
‡‡ $P = 0.12$ when compared to control data using a Fisher
15 exact test.

Table 5. Pathological Disease in SJL/J Mice with R-EAE After Treatment with SCH 94.03

		Pathological Score		
	Treatment	n	Demyelination	Meningeal inflammation
5	SCH94.03	14	24.6 ± 3.6*	18.7 ± 3.6*
	Control ‡	19	39.3 ± 6.0	31.8 ± 5.3

SJL/J mice with R-EAE were treated as described in the Table 1 legend. The pathological scores were determined by a semi-quantitative morphological analysis and represent the percentage of spinal cord quadrants with the indicated pathological abnormality. One mouse treated with control IgM had minimal gray matter inflammation, whereas all other animals showed no inflammation in spinal cord gray matter. The data are from 4 independent experiments and are presented as the mean \pm SEM where nd indicates the number of mice. * $P < 0.05$ when compared to control data. ‡ Combined data from mice treated with control IgM or PBS as described in the Table 4 legend.

Thus, it is reasonable to predict that autoantibodies, such as SCH94.03, play a critical role in stopping an immune-mediated process of demyelination in CNS diseases.

Two potential mechanisms can be proposed by which Abs promote remyelination. First, Abs might inhibit some pathogenic component of the disease process, such as virus activity, an immune response which directly suppresses remyelination. If the disease outcome is based upon a balance between tissue destruction and repair, inhibition of pathogenic components would allow a physiological repair response to predominate. Experimental and clinical evidence support this hypothesis. Spontaneous CNS remyelination is seen in MS patients and several experimental models of CNS demyelination as well as described herein, demonstrating spontaneous remyelination in control mice. This indicates that remyelination is a normal physiological response to myelin damage. In addition, treatment of mice chronically infected with TMEV with various immunosuppressive regimens promotes remyelination, but does not decrease demyelination, indicating that there is an immunological component which inhibits remyelination. Immunological function studies reported in Miller et al., International Immunology, (1996) 8, pp 131-141, The disclosure of which is incorporated herein by reference, indicate that animals treated with SCH94.03 had similar numbers of B and T (both CD4+ and CD8+) cells in their spleens compared to control animals, had similar *in vitro* splenocyte proliferative responses to mitogens and antigens, and mounted comparable Ab responses to both T cell-dependent and T cell-independent antigens. See Table 6, below. However, there was a 2 to 3 fold reduction in the number of CD4 and CD8 T cells infiltrating the CNS of mice treated with the mAb 94.03. Treatment with 94.03 also suppresses the humoral immune

response to a T cell-dependent antigen in chronically infected mice. Immunohistochemical staining showed that 94.03 labeled MHC Class II positive dendrite cells in peripheral lymphoid organs. These results thus suggest
5 that one of the mechanisms by which Mab SCH94.03 maybe promoting remyelination is by inhibiting a pathogenic immune response.

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Table 6. FCM analysis of mononuclear cells infiltrating the CNS of chronically infected SJL/J mice.

Treatment	N	Total n. of surface marker positive CNS-infiltrating mononuclear cells ($\times 10^5$) ^a			
		CD5 ⁺	CD4 ⁺	CD8 ⁺	CD45R(B220) ⁺
PBS	10	6.2 \pm 0.8	3.0 \pm 0.4	2.4 \pm 0.3	0.4 \pm 0.1
Control IgM	12	5.0 \pm 0.6	3.0 \pm 0.4	1.7 \pm 0.2	0.2 \pm 0.0
SCH94.03	12	2.3 \pm 0.4 ^b	1.4 \pm 0.2 ^c	0.8 \pm 0.2 ^b	0.1 \pm 0.0 ^d

5

SJL/J mice chronically infected with TMEV were injected i.p. with a total dose of 0.5 mg SCH94.03, control IgM or an equivalent volume PBS, divided into twice weekly doses for 5 weeks. For control IgM, MOPC104E and XXMEN-OE5 were used. The data are a composite of independent experiments and are presented as the mean \pm SEM, where N indicates the number of mice.

10

^aCell numbers were calculated by multiplying the percentage of positive cells assessed by FCM with the total number of mononuclear cells isolated from brain and spinal homogenates of individual mice by Percoll gradient separation.

15

^bP<0.00001 when compared with combined control IgM and PBS data.

^cP<0.00005 when compared with combined control IgM and PBS data.

⁴P<0.007 when compared with combined control IgM and PBS data.

"open" endothelium, or perhaps via an unidentified active transport mechanism.

Treatment of Demyelinating Diseases

The results of the experiments described herein have
 5 practical applications to multiple sclerosis (MS), EAE,
 and other related central nervous system demyelinating
 disorders. Rare examples of spontaneous CNS-type
 remyelination ("shadow plaques") are found in MS and
 occasional peripheral nervous system (PNS)-type
 10 remyelination is found in demyelinated spinal cord
 plaques near the root entry zone. Oligodendrocytes are
 infrequent at the center of the chronic plaques in MS but
 they appear to proliferate at the periphery of plaques,
 where they are associated with abortive remyelination.
 15 The process of remyelination may correlate with the
 spontaneous remission and improvements observed
 clinically in MS. These clinical observations indicate
 that new myelin formation is possible in MS. The
 remyelination that has been stimulated in mice with TMEV-
 20 induced demyelination by using a mAb holds promise for
 therapeutic applications in multiple sclerosis.

Of importance clinically is the question of whether
 morphologic regeneration of thin myelin sheaths
 contributes to functional recovery. Computer simulations
 25 indicate that new myelin formation even by
 inappropriately thin sheaths improves impulse conduction.
 Since the axon membrane of normally myelinated fibers is
 highly differentiated, it is necessary for sodium
 channels to be present at high density at the node of
 30 Ranvier to propagate saltatory conduction. Experimental
 evidence suggests that newly formed nodes do develop the
 required high sodium channel density as demonstrated by
 saxitoxin binding. Data to date suggest that
 remyelination even by inappropriately thin myelin
 35 improves conduction in a previously demyelinated axon.

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Therefore, any strategy to promote this morphologic phenomenon has the potential of producing functional recovery.

The data presented herein demonstrates, for the first
5 time, that administration of a monoclonal antibody to a
mammal is capable of stimulating remyelination of central
nervous system axons *in vivo*. Specifically, treatment of
chronically infected TMEV-infected mice with as little as
10 μ g of SCH94.03 resulted in a 4- to 5-fold increase in
the total area of CNS myelination compared to mice
treated with a control mAb.

Thus, as a result of the experiments described herein,
the method of the present invention can be used to treat
mammals, including humans and domestic animals, afflicted
15 with demyelinating disorders, and to stimulate
remyelination of the CNS axons. As described herein, an
effective amount of the monoclonal antibody can be
administered by conventional routes of administration,
and particularly by, intravenous (iv) or intraperitoneal
20 (ip) injection. An effective amount of the antibody can
vary depending on the size of the mammal being treated,
the severity of the disease, the route of administration,
and the course of treatment. For example, each dose of
mAb administered can range from approximately 0.5 mg/kg
25 to approximately 400 mg/kg, with the preferred range from
approximately 0.5 mg/kg to approximately 250 mg/kg. It
is important to note that a dose as low as 10 μ g (0.5
mg/kg) was effective in promoting remyelination of CNS
axons in mice. The dose of mAb will also depend on the
30 route of administration. For example, an iv dose
administered to mice was 0.5 mg/kg, and an ip dose was
5.0 mg/kg. The course of treatment includes the
frequency of administration of the mAb (e.g., daily,
weekly, or bi-weekly) and the duration of the treatment
35 (e.g., four weeks to four months). Thus, for example, a

larger amount of mAb can be given daily for four to five weeks, as opposed to a smaller amount of mAb given for four months.

The effectiveness of the amount of the monoclonal antibody being administered can be assessed using any number of clinical criteria, for example, as described in Example 3, including overall appearance of the mammal, the activity of the mammal and the extent of paralysis of the mammal. The effectiveness of the amount of monoclonal antibody necessary to induce remyelination in humans can also be assessed in a double blinded controlled trial. Patients with fixed neurological deficits from demyelinating disease can be treated with monoclonal antibody or controls. Improvement in isometric muscle strength as detected by quantitative biomechanics muscle testing could be used as the primary therapeutic end-point.

Additionally, the monoclonal antibody may be genetically altered, e.g. "humanized" by the substitution of human antibody nucleotide sequences in non-variable regions of the murine mAb to reduce immunogenicity.

In addition to *in vivo* methods of promoting remyelination, *ex vivo* methods of stimulating remyelination in CNS axons are also encompassed by the present invention. For example, the monoclonal antibody may be used *in vitro* to stimulate the proliferation and/or differentiation of glial cells, such as oligodendrocytes, as described in Example 2. These exogenous glial cells can then be introduced into the CNS of mammals using known techniques. Remyelination of CNS axons would be increased by increasing the number of endogenous glial cells present (glial cells, such as oligodendrocytes play a critical role in the production of myelin).

In vitro methods of producing glial cells, or stimulating the proliferation of glial cells from mixed culture (e.g., rat optic nerve cell, or rat brain cell cultures) are also encompassed by this invention. For example, 5 cells obtained from rat optic nerve, or rat brain, containing glial cells, are cultured as a mixed culture under conditions sufficient to promote growth of the cells. An effective amount of mAb capable of promoting remyelination of CNS axons, such as SCH94.03, is then 10 added to the mixed culture of cells and maintained under conditions sufficient for growth and proliferation of cells. The mAb stimulates the proliferation of glial cells cultured in the presence of the mAb is increased, relative to the proliferation of glial cells grown in the 15 absence of the mAb.

The monoclonal antibodies for use in the methods of the present invention can be, and are preferably, administered as medicaments, i.e., pharmaceutical compositions. An effective amount of the monoclonal 20 antibody can thus be combined with, or diluted with, an appropriate pharmaceutically acceptable carrier, such as a physiological buffer, or saline solution.

The pharmaceutical compositions used in the methods of this invention for administration to animals and humans 25 comprise the monoclonal antibodies in combination with a pharmaceutical carrier or excipient.

The medicament can be in the form of tablets (including lozenges and granules), dragees, capsules, pills, ampoules or suppositories comprising the compound of the 30 invention.

Advantageously, the compositions are formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredients. Tablets, coated tablets, capsules,

ampoules and suppositories are examples of preferred dosage forms according to the invention. It is only necessary that the active ingredient constitute an effective amount, i.e., such that a suitable effective dosage will be consistent with the dosage form employed in single or multiple unit doses. The exact individual dosages, as well as daily dosages, will, of course, be determined according to standard medical principles under the direction of a physician or veterinarian.

- 10 The monoclonal antibodies can also be administered as suspensions, solutions and emulsions of the active compound in aqueous or non-aqueous diluents, syrups, granulates or powders.

Diluents that can be used in pharmaceutical compositions (e.g., granulates) containing the active compound adapted to be formed into tablets, dragees, capsules and pills include the following: (a) fillers and extenders, e.g., starch, sugars, mannitol and silicic acid; (b) binding agents, e.g., carboxymethyl cellulose and other cellulose derivatives, alginates, gelatine and polyvinyl pyrrolidone; (c) moisturizing agents, e.g., glycerol; (d) disintegrating agents, e.g., agar-agar, calcium carbonate and sodium bicarbonate; (e) agents for retarding dissolution, e.g., paraffin; (f) resorption accelerators, e.g., quaternary ammonium compounds; (g) surface active agents, e.g., cetyl alcohol, glycerol monostearate; (g) adsorptive carriers, e.g., kaolin and bentonite; (i) lubricants, e.g., talc, calcium and magnesium stearate and solid polyethylene glycols.

- 30 The tablets, dragees, capsules and pills comprising the active compound can have the customary coatings, envelopes and protective matrices, which may contain opacifiers. They can be so constituted that they release the active ingredient only or preferably in a particular

part of the intestinal tract, possibly over a period of time. The coatings, envelopes and protective matrices may be made, for example, from polymeric substances or waxes.

- 5 The diluents to be used in pharmaceutical compositions adapted to be formed into suppositories can, for example, be the usual water-soluble diluents, such as polyethylene glycols and fats (e.g., cocoa oil and high esters, [e.g., C₁₄-alcohol with C₁₆-fatty acid]) or mixtures of these
10 diluents.

The pharmaceutical compositions which are solutions and emulsions can, for example, contain the customary diluents (with, of course, the above-mentioned exclusion of solvents having a molecular weight below 200, except
15 in the presence of a surface-active agent), such as solvents, dissolving agents and emulsifiers. Specific non-limiting examples of such diluents are water, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene
20 glycol, 1,3-butylene glycol, dimethylformamide, oils (for example, ground nut oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitol or mixtures thereof.

For parental administration, solutions and suspensions
25 should be sterile, e.g., water or arachis oil contained in ampoules and, if appropriate, blood-isotonic.

The pharmaceutical compositions which are suspensions can contain the usual diluents, such as liquid diluents, e.g., water, ethyl alcohol, propylene glycol, surface
30 active agents (e.g., ethoxylated isostearyl alcohols, polyoxyethylene sorbitols and sorbitan esters), microcrystalline cellulose, aluminum methahydroxide, bentonite, agar-agar and tragacanth, or mixtures thereof.

The pharmaceutical compositions can also contain coloring agents and preservatives, as well as perfumes and flavoring additions (e.g., peppermint oil and eucalyptus oil), and sweetening agents, (e.g., saccharin and aspartame).

The pharmaceutical compositions will generally contain from 0.5 to 90% of the active ingredient by weight of the total composition.

In addition to the monoclonal antibodies, the pharmaceutical compositions and medicaments can also contain other pharmaceutically active compounds.

Any diluent in the medicaments of the present invention may be any of those mentioned above in relation to the pharmaceutical compositions. Such medicaments may include solvents of molecular weight less than 200 as the sole diluent.

It is envisaged that the monoclonal antibodies will be administered perorally, parenterally (for example, intramuscularly, intraperitoneally, subcutaneously, transdermally or intravenously), rectally or locally, preferably orally or parenterally, especially perlingually, or intravenously.

The administered dosage rate will be a function of the nature and body weight of the human or animal subject to be treated, the individual reaction of this subject to the treatment, type of formulation in which the active ingredient is administered, the mode in which the administration is carried out and the point in the progress of the disease or interval at which it is to be administered. Thus, it may in some case suffice to use less than a minimum dosage rate, while other cases an upper limit must be exceeded to achieve the desired

results. Where larger amounts are administered, it may be advisable to divide these into several individual administrations over the course of the day.

The present invention will be better understood from a
5 consideration of the following examples, which describe
the preparation of compounds and compositions
illustrative of the present invention. It will be
apparent to those skilled in the art that many
modifications, both of materials and methods, may be
10 practiced without departing from the purpose and intent
of this disclosure.

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EXAMPLESExample 1Monoclonal Antibody Production, Screening and Purification5 Animals

Spleens of two SJL/J mice (Jackson Laboratories, Bar Harbor, ME) that had been injected twice with spinal cord homogenate (SCH) in incomplete Freund's adjuvant were used as the source of B cells for fusion and hybridoma production. Splenocytes were fused with NS-1 myeloma cells using polyethylene glycol, and viable cell fusions were selected with hypoxanthine-aminopterin-thymidine (HAT) media and cloned by limiting dilution as described (Katzmann, J.A. et al., Proc. Nat. Acad. Sci. USA, 78:162-166 (1981)).

ELISAs

Hybridoma supernatants from viable Ig-producing clones were screened for binding to SCH by an enzyme-linked immunosorbant assay (ELISA). The following antigens were used for screening mAbs: SCH - (10 μ g) reconstituted in carbonate-bicarbonate buffer (pH 8.53), MBP - (1 μ g) dissolved in PBS, GC (1 μ g) dissolved in absolute alcohol, PLP (1 μ g) dissolved in water. PLP was provided by Dr. W. Macklin (UCLS) who has published a solid phase immunoassay for PLP. For SCH, MBP or GC ELISA, Immuno II plates were coated with prepared antigen (100 μ l/well) which was incubated overnight at 4°C. The following day well were washed in PBS and blocked with PBS + 1% serum for 1 hour at room temperature. Plates were washed again in PBS and serial dilutions of primary Ab diluted in PBS/0.1% BSA were added and incubated at room temperature for 2 hours. Plates were washed in PBS/0.05% Tween and appropriate secondary Ab conjugated to alkaline phosphatase (1:1000 in PBS 0.1% BSA) was added. Plates were incubated at 37°C for 2 hours, washed in PBS 0.05%

Tween, and the substrate (Sigma 104 Phosphatase Substrate Tablet in 5 ml diethanolamine buffer) was added for 30 min. The reaction was terminated with 50 μ l of 1 N NaOH. The plates were read on a Dynatech ELISA plate reader.

5 Ascites production

The hybridomas chosen for treatment experiments were injected into pristane-treated BALB/c mice for ascites production. Hybridomas were also grown in RPM1-1640 media supplemented with 10% fetal bovine serum for IgM production. IgM mAbs were purified by either ammonium sulfate precipitation and gel filtration on a Sephacryl S-400 HR (Sigma) column for the initial transfer experiments, or by affinity chromatography using goat anti-mouse IgM (μ -chain specific; Jackson ImmunoResearch, West Grove, PA) coupled to Reacti-Gel 6X matrix (Pierce, Rockford, IL) for later transfer experiments.

Example 2

In Vitro Testing of Monoclonal Antibodies

Selection of mAbs that promote glial cell proliferation

20 The ability of the mAbs to promote proliferation of glial cells *in vitro* was tested. Glial cells isolated from rat brain or optic nerves were seeded in Falcon Microtest II plates at a concentration of 2×10^4 cells per well in 0.1 ml of DME. Whole serum (SCH, IFA, MBP, GC, MBP/GC, PBS or PLP), purified Ig or mAb, was serially diluted and 0.1 ml aliquot was added to cells and assayed in triplicate. Three days later ^3H -thymidine was added ($1 \mu\text{Ci/ml}$) and cells were harvested after 17 hours with an automated cell harvester (Mash II Harvester). To document identity of cells proliferating (i.e., astrocytes, progenitor glial cells, macrophages), selected cultures after exposure to ^3H -thymidine, were incubated with appropriate Ab specific for cell type followed by ABC immunoperoxidase technique. After reaction of Hanker- Yates reagent, the slides were immersed in Ilford K2

nuclear emulsions, exposed for 4 days at 4°C and developed.

mAb 94.03 and 94.32 induce proliferation of mixed rat optic nerve brain cultures

- 5 One- to two-day-old rats were killed with ether. Through careful dissection, optic nerves were removed from the optic nerve chiasm to the eye. Nerves were transferred to centrifuge tubes containing 2 mls of DMEM. An equal volume of 0.25% trypsin was added and incubated to 37°C
- 10 in a water bath for 45 min. 0.2 ml of FCS was added to terminate trypsinization. Nerves were passed through a sterile needle and syringe (gauge no. 21) and then centrifuged at 1400 rpm for 10 minutes. The cell count was adjusted to provide concentration of 5×10^5 cells/100
- 15 μ l of media in 24-well trays in DMEM + 0.5% FCS. After 12 to 16 hours, appropriate antibodies or growth media were added as per experimental protocols.
- Brains of 1-2 day old rats were removed and placed in Hank's Balanced Salt Solution with 10 mM HEPES buffer
- 20 (HBSS/H), approximately 1-2 ml per brain. The brain stem, cerebellum, and midbrain was discarded whereas the forebrain was minced with a bent syringe. The tissue was further disrupted by repeated passage through a 10 ml pipet and transferred to a 50 ml conical tube. The
- 25 tissue suspension was shaken on a rotary shaker (75 rpm) for 30 min at 37°C. Trypsin was added to a final concentration of 0.125% and the suspension was shaken for an additional 60 minutes. Trypsin digestion was stopped by adding FCS (10%). The cell suspension was passed
- 30 sequentially through 120 and 54 μ m Nytex, centrifuged, resuspended in serum-free medium with 10% FCS, and filtered again through 54 μ m Nytex. Serum-free media was DMEM with 3.7 g/l sodium bicarbonate, 6.0 g/l glucose, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 5 μ g/ml
- 35 insulin, 5 μ g/ml streptomycin. The cells were counted, plated onto uncoated tissue culture flasks or plates at

5x10⁴ cells/cm² and cultured at 37°C in 5% CO₂. The media was changed after 72 hours, and every 48 hours thereafter. On day 8 after culture initiation, the media was aspirated and replaced by SFM with various supplements (for example, antibody). For most experiments, the cells were grown for an additional 48 hours before harvesting. Cells were pulsed with [³H]thymidine (5 µCi/ml) for the final 1824 hours of culture.

10 Western Blot Procedure

Antigens were denatured and solubilized by heating at 100°C in sodium dodecyl sulfate (SDS) sample buffer. Samples were electrophoresed on stacking and separating gels containing 4.75% and 12.0% acrylamide at 200 volts.

15 After electrophoresis, gels and nitrocellulose membranes were equilibrated for 30 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.1-8.3). All steps were done at room temperature. Gels were electroblotted for either 1 hour at 100V or overnight at

20 30V using the Bio-Rad Mini Trans-blot apparatus. The nitrocellulose membrane was cut into strips and washed, 3X TBS (100 mM NaCl, 50 mM Tris, pH 7.6) with 0.03% Tween 20. Nitrocellulose strips were blocked (TBS with 3% non-fat milk and 0.03% Tween 20) for 2-4 hours, washed 3X,

25 and incubated with primary Ab or antisera (diluted in blocking buffer) for 4 hours or overnight. After primary Ab incubation, strips were washed 3X, incubated with either biotin- or alkaline phosphate-labelled secondary Ab (diluted in blocking buffer) for 2 hours, washed 3X,

30 and incubated with alkaline-phosphatase labeled-streptavidin (diluted in blocking buffer) for 2 hours if the biotin system is used. Nitrocellulose strips were washed 4X (final wash in TBS without Tween 20) and incubated with substrate solution (0.165 mg/ml BCIP and

35 0.33 mg/ml NBT in 100 mM sodium chloride, 100 mM Tris, 5 mM MgCl₂, pH 9.5) until sufficient color developed

Cell lines or mixed brain cultures were lysed in 1X SDS reducing sample buffer (2.3% SDS, 10% 2-ME, 0.125 M Tris, 20% glycerol) and heated to 85°C for 15 minutes. Nucleic acids were sheared by repeated passage of lysate through 21-27-gauge needles. Lysate proteins were separated on a 12% acrylamide reducing gel, transferred to nitrocellulose membranes, and blotted with various antibodies as previously described.

Promotion of CNS Remyelination Using a Monoclonal Antibody
Virus

25 In vitro TMEV neutralization assay

Viral plaque assays were done as previously described (Patick, A.K., et al., J. Neuropath. Exp. Neurol., 50:523-537 (1991)). To assess neutralization, aliquots of TMEV (200 PFU/ml) were incubated with various concentrations of Ab for 1 hour at room temperature prior to plating onto confluent L2 cells. As a positive control, serum from susceptible mice chronically infected with TMEV was used. Under the assay conditions described above, a serum dilution of 1:34,000 gave 50%

neutralization, which corresponded to an estimated 20 ng/ml of TMEV-specific Abs, assuming a total serum Ig concentration of 15 mg/ml, and a TMEV-specific fraction of 5%.

5 Demyelination protocol

Demyelination was induced in female SJL/J mice, ages four to six weeks, from the Jackson Laboratory, Bar Harbor, ME. Mice were inoculated intracerebrally with 2×10^5 plaque-forming units of DA virus in a volume of 10 μ l.

- 10 Mice infected chronically with TMEV (4 to 6 months following infection) were assigned randomly to groups of treatment.

Treatment protocol and clinical disease assessment

Chronically infected mice were given either

- 15 intraperitoneal (ip) or intravenous (iv) injections of mAb twice weekly for 4-5 weeks. At each treatment injection, mice were assessed clinically by three criteria: appearance, activity, and paralysis. A score for each criterion was given ranging from 0 (no disease) to 3 (severe disease). For appearance, 1 indicated minimal change in coat, 2 indicated a severe change (incontinence and stained coat). For activity, 1 indicated decreased spontaneous movements (minimal ataxia), 2 indicated moderate slowing (minimal spontaneous movements), and 3 indicated severe slowing (no spontaneous movement). For paralysis, 0.5 indicated a spastic extremity, 1 indicated a paralyzed extremity, 1.5 indicated two or more spastic extremities, 2 indicated two paralyzed extremities (unable to walk), 2.5 indicated no righting response, and 3 indicated three or four paralyzed extremities (moribund). The total score for each mouse was the cumulative total from each criterion (maximum of 9). As the clinical score was an ordinal, but not a cardinal scale, the change in clinical score to assess clinical disease was used. The clinical

assessment data were not disclosed until after the morphological assessment of remyelination was completed.

Light and electron micrograph preparation and assessment of remyelination

- 5 Preparation of light and electron microscopy sections and morphological assessment of remyelination were done. Briefly, treated mice were anesthetized with pentobarbital (0.2 mg ip), exsanguinated by cardiac puncture, and filled by intracardiac perfusing with
- 10 Trump's fixative (100 mM phosphate buffer, pH 7.2, with 4% formaldehyde and 1.5% glutaraldehyde). The entire spinal cord was removed carefully from the spinal canal, and sectioned into 1 mm transverse blocks. Every third block was post-fixed in 1% osmium tetroxide and embedded
- 15 in Araldite (Polysciences, Warrington, PA). One micron sections from each block were cut and stained with p-phenylenediamine. On each section, remyelination was quantitated using a Zeiss interactive digital analysis system (ZIDAS) and camera lucida attached to a Zeiss
- 20 photomicroscope (Carl Zeiss Inc., Thornwood, NY). Abnormally thin myelin sheaths relative to axonal diameter was used as the criterion for CNS remyelination. Ten spinal cord sections from each mouse were examined; this corresponded to 8-9 mm² of white matter examined per
- 25 mouse. To avoid bias, slides were coded and quantitation was done without knowledge of the treatment groups.

Myelin thickness and axonal diameter measurements and quantitation of myelination axons

- Electron micrographs of normal and remyelinated axons
- 30 from plastic-embedded spinal cord sections were imaged with a Hamamatsu video camera, digitized, and analyzed using an IBAS 2000 Image Analysis System (Kontron, Munich, Germany). The Axonal cross-sectional area with and without the myelin sheath was measured, and
- 35 equivalent circle calculations were used to determine the

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axonal diameter and myelin sheath thickness. For myelinated axon quantitation, the number of myelinated axons in lesions from plastic-embedded spinal cord sections were counted using the analysis system described above attached to an Axiophot microscope (Carl Zeiss, Inc.). 17 remyelinated and 15 demyelinated lesions in spinal cord sections from animals treated with mAb SCH94.03, control IgM, or buffer only were analyzed. This corresponded to 0.6mm² of remyelinated area and 0.8 mm² of demyelinated area. The criterion for selection of a lesion as demyelinated was the presence of substantial demyelination with minimal repair, whereas remyelinated lesions were chosen based upon the presence of almost complete remyelination throughout the lesion.

15 Immunostaining

Rat 5.5B8 glial cells were grown on poly-D/L-lysine-coated chamber slides in Dulbecco's modified Eagle's medium (DMED) supplemented with 1.5 g/L D-glucose, 30 nM SeO₂, 15 nM triiodothyronine, 10 ng/ml biotin, 100 µM ZnCl₂, 50 µg/ml gentamicin, and 10% fetal bovine serum. All staining steps were done at room temperature. For surface staining, slides were briefly rinsed with PBS, and cells were lightly fixed with 1% formaldehyde in PBS for 10 minutes to prevent cell detachment during subsequent staining steps. For cytoplasmic staining, slides were rinsed twice in PBS and either air dried for 1 hour or incubated with 0.1% Triton X-100 in PBS for 10 minutes. Cells were blocked in 2% BSA for 30 min, washed, incubated with control IgM or mAb SCH94.03 (10 µg/ml in 1% BSA) for 1 hour, and washed extensively with PBS. After fixation with 4% paraformaldehyde for 15 min, slides were incubated with fluorescein-labeled goat anti-mouse IgM (Jackson ImmunoResearch) for 1 hour, washed with PBS, coverslipped with 10% MOWIOL® (Hoechst) in 100 mM Tris, 25% glycerol, pH 8.5 with 25 µg/ml 1,4-diazobicyclo-[2.2.2]-octane (DABCO) to prevent fading,

and allowed to set overnight in the dark. For frozen tissue sections, fresh neonatal rat, adult mouse, or autopsied human cortical brain tissue was quick frozen in isopentane chilled in liquid nitrogen prior to liquid
5 nitrogen storage. Frozen sections (10 μ m) were transferred onto gelatinized glass microscope slides, air dried for 4-8 hours, and stored at -70°C. Prior to immunostaining, slides were placed at room temperature overnight. The immunoperoxidase staining protocol was
10 similar that described above, using the ABC immunoperoxidase reagent (Vector Laboratories, Burlingame, CA), developed with 1.5 mg/ml Hanker-Yates reagent (p-phenylene diamine-procatechol) in 50 mM Tris, pH 7.6 with 0.034% H₂O₂, counterstained with Mayer's
15 hematoxylin, and mounted with Permount (Fischer Scientific, Pittsburgh, PA).

Data Analysis

A modified cumulative rank sum test (O'Brien, P.C., Biometrics, 40:1079-1087 (1984)) was used to compare
20 remyelination between treatment groups. This statistical test takes into account several numerically unrelated parameters of therapeutic effectiveness, and is used routinely for clinical trial efficacy assessment. Parallel analyses using a standard unpaired Student's t-
25 test to compare individual parameters of remyelination gave equivalent results. Comparisons of disease severity and correlation significance were determined by a one-way analysis of variance (ANOVA). Statistical analyses were done with either the SigmaStat (Jandel Scientific, San
30 Rafael, CA) or EXCEL (Microsoft Corporation, Redmond, WA) software programs. Calculated values were considered significant when p was < 0.05.

Example 4

1. Hybridoma culture and determination of Ig isotype

A2B5, HNK-1, and XXMEN-OE5 (anti-bacterial lipopolysaccharide) hybridomas were purchased from American Type Culture Collection (Rockville, MD). O1 and O4 hybridomas were the gift of Dr. S.E. Pfeiffer

5 (University of Connecticut, Farmington, CT). Hybridomas were cultured in RPMI 1640 containing 10% fetal calf serum (HyClone, Logan, UT) and 2×10^{-2} mM β -mercaptoethanol. IgM concentrations of the supernatants were determined by a μ -chain-specific capture ELISA With

10 purified MOPC104E (Sigma, St. Louis, MO) as the standard. To determine the IgM isotype of mAbs O1, O4, and A2B5, Mouse Monoclonal Antibody Isotyping Kit (Gibco, Grand Island, NY) was used.

2. mRNA isolation and cloning of Ig variable region

15 Poly(A)⁺ RNA was isolated from hybridoma cells by oligo(Dt)-cellulose chromatography using the Micro-Fast Track kit (Invitrogen, San Diego, CA). Ig heavy and light chain variable region cDNAs were cloned by the 5'-rapid amplification of cDNA ends (RACE) method using the

20 5'-AmpliFINDER™ RACE kit (Clontech, Palo Alto, CA). Briefly, first strand cDNA was synthesized using an oligo Dt primer. An anchor oligonucleotide was ligated to the 3' end of the first strand cDNA, and variable region cDNAs were amplified by polymerase chain reaction using

25 primers corresponding to the anchor sequence and constant region-specific primers for the μ ($C\mu$) or κ ($C\kappa$) chains described previously [Miller et al., J. Immunol., 154 (1995), 2460-2469].

3. Sequencing and analysis

30 Amplified cDNA products were purified from agarose gel after electrophoresis and directly subcloned into pCRII using the TA cloning kit (Invitrogen). Both strands of the insert were sequenced using automated DNA sequencer (Applied Biosystems model 373A, Mayo Molecular Biology

35 Core Facility). For nucleotide sequence homology

searches, the FastA program (GCG program, version 8) was used [Devereux, J. et al., *Nucleic Acids Res.*, 12 (1984), 387-395].

4. *Direct ELISA to determine polyreactivity*

- 5 HNK-1 was shown previously to be polyreactive by Western blots [McGarry et al., *supra*]. Therefore, the polyreactivity of O1, O4 and A2B5 was tested by direct ELISA. Human RBC spectrin, bovine myosin (heavy chain), mouse albumin, mouse hemoglobin, mouse transferrin, hen
10 egg lysozyme, rabbit actin, rabbit myelin basic protein, and keyhole limpet hemocyanin (KLH) were purchased from Sigma. Proteins were tested for purity by SDS-polyacrylamide gel electrophoresis. The chemical hapten trinitrophenyl (TNP) was coupled to bovine serum albumin
15 (BSA) [Miller et al., 1995, *supra*]. Protein antigens were used at 5 μ g/ml, and hapten was used at 2 μ M. The proteins and hapten-BSA antigens were coated onto polystyrene or polyvinylchloride microtiter plates in 0.1 M carbonate buffer, pH 9.5, for 18 hours at 4°C. Coated
20 plates were blocked with PBS containing 5% nonfat dry milk and 0.05% Tween 20 for 2 hours at room temperature, and incubated with mAbs diluted in blocking buffer (2 μ g/ml) for 4 hours at room temperature. TEPC183 (Sigma) and XXMEN-OE5 IgM mAbs were used as control antibodies.
25 Bound IgM was detected with biotinylated goat anti-mouse IgM (μ chain specific; Jackson ImmunoResearch, West Grove, PA) followed by alkaline phosphatase conjugated to streptavidin, with p-nitrophenylphosphate as the chromogenic substrate. Absorbance was determined at 405
30 nm.

Results

Nucleotide sequences of variable region cDNA including leader peptide were compared with published sequences of germline genes, mouse myeloma and natural autoantibodies.

1. Heavy chain variable region cDNA sequences

O1 V_H was identical with unrearranged V_H segment transcripts A1 and A4 [Yancopoulos et al. Cell, 40 (1985), 271-281], which belong to V_H558 family (Figure 12). The O1 D segment was relatively short and contained four nucleotides derived from germline SP2 gene family (common sequence to DSP2.3, 2.4 and 2.6) [Kurosawa et al., J. Exp. Med., 155 (1982), 201-218]. The D segment for O1 was dG and dC rich in the 5' end, probably representing non-coded (N) nucleotides inserted by terminal deoxynucleotide transferase (TdT) during V-D-J recombination. O1 displayed sequence identity with germline J_H1 [Sakano et al., Nature, 286 (1980), 676-683], except for one nucleotide (GTC for GTT in the germline), which did not result in an amino acid substitution.

Compared with the germline BALB/c V_H101 [Kataoka et al., J. Biol. Chem., 257 (1982), 277-285], O4 V_H showed three nucleotide differences in the V_H coding region (Figure 13), all of which resulted in amino acid substitutions. Compared to natural autoantibody D23 [Baccala et al., Proc. Natl. Acad. Sci. USA, 86 (1989), 4624-4628], which is encoded by germline V_H101, O4, V_H showed two nucleotide differences with amino acid substitutions in the V_H coding region. Compared with germline V_H101, HNK-1 V_H showed six nucleotide differences and four amino acid differences in the V_H coding region (Figure 13). Compared to natural autoantibody D23, HNK-1 V_H showed five nucleotide differences and three amino acid differences in the V_H coding region. D23 had three nucleotide differences when compared with germline V_H101; all differences were also seen in the O4 and HNK-1 V_H. The O4 D segment contained five nucleotides and the HNK-1 D segment contained 13 nucleotides derived from germline DFL16.1 gene [Kurosawa et al., J. Exp. Med., 155 (1982),

201-218]. The HNK-1 D segment had one dG residue in the 5' end and four dG residues in the 3' end, which probably represent N nucleotides inserted by TdT during V-D-J recombination. The heavy chain joining region of O4
 5 corresponded to germline J_H4 [Sakano et al., *supra*]. The heavy chain joining region of HNK-1 corresponded to germline J_H4 beginning with the fifth codon.

The A2B5 V_H showed seven nucleotide and four amino acid differences in its coding region in comparison with the
 10 germline V1 (also called T15 and S107) [Crews et al., Cell, 25 (1981), 59-66; Siu et al., J. Immunol., 138 (1987), 4466-4471] (Figure 14). The heavy chain joining region of A1B5 corresponded to germline J_H3 beginning with the third codon [Sakano et al., *supra*].

15 2. Light chain variable region cDNA sequences

Since all the hybridomas produced IgM _x antibodies as determined by isotyping assay, a C_x primer was used for polymerase chain reaction. O1 and O4 light chain variable region cDNA sequences were identical (Figure
 20 15). The V_x segments of O1 and O4 were identical with natural autoantibody E7 [Baccala et al., *supra*], and showed only one silent nucleotide difference when compared with myeloma MOPC21 [Hamlyn et al., Nucleic Acids Res., 9 (1981), 4485-4494]. The J_x segment of HNK-
 25 1 showed sequence identity with J_x2.

The genomic germline gene which encodes the V_x segment of A2B5 (Figure 17) is unknown, but belongs to the V_x19 group [Potter et al., Mol. Immunol., 19 (1982), 1619-1630]. The V_x segment of A2B5 was identical with the V_x
 30 segment from hybridomas H220-11, H230-2, H230-5 and H250-6 [Caton et al., J. Immunol., 147 (1991), 1675-1686] except for two nucleotide changes, one of which resulted in an amino acid substitution (data not shown). The V_x

segments of H220-11, H230-2, H230-5 and H250-6 are identical to each other. The J_x segment of A2B5 was identical with J_x5 [Max et al., J. Biol. Chem., 256 (1981), 5116-5120; Sakano et al., *supra*] except for one
 5 nucleotide which resulted in an amino acid substitution.

3. Direct ELISA

To assess the polyreactivity of the O1, O4, and A2B5, binding of mAbs to a panel of defined antigens was determined by ELISA (Figure 18). O1 reacted with human
 10 RBC spectrin. O4 reacted with human RBC spectrin, bovine myosin, mouse hemoglobin, rabbit actin, and TNP-BSA. A2B5 and the two control IgM_x mAbs did not react with this panel of antigens.

4. Utility

15 The enormous diversity in the Ig variable region is due primarily to combinations of multiple germline coding gene segments. Different primary structures are produced by recombination of V,D,J (heavy chain) or V,J (light chain) gene segments. Assuming the random
 20 association of heavy and light chains to form a complete antibody molecule, the number of different molecules is estimated to be 1.6×10^7 [Max et al., Fundamental Immunology, Raven Press, NY, 1993, pp. 315-382]. Somatic mutation during the process of antigen challenge provides
 25 even further diversity and specificity. In contrast to the majority of Igs produced following antigen challenge, natural autoantibodies are encoded directly by germline genes with no or few mutations. Natural autoantibodies are present in sera of healthy humans and rodents
 30 [Dighiero et al., J. Immunol., 131 (1983), 2267-2272; Guilbert et al., J. Immunol., 128 (1982), 2279-2287; Hartman et al., Mol. Immunol., 26 (1989), 359-370]. These natural autoantibodies are polyreactive, capable of binding to a variety of structurally unrelated antigens
 35 [Avrameas et al., Mol. Immunol., 30 (1993), 1133-1142].

The physiologic function of natural autoantibodies is unknown. However, by interacting with many self constituents, these natural autoantibodies and their targets are believed to establish a vast network whereby the immune system can participate in general homeostasis.

These results provide evidence based on Ig variable region cDNA sequences that three of the four OL-reactive IgM_x mAbs (O1, O4 and HNK-1) have characteristics of natural autoantibodies. The J_H segments of O4 and HNK-1, and the J_x segments of O1, O4 and HNK-1 are encoded by unmutated germline Ig genes. The J_x segment of O1 has only one silent nucleotide change. O1 V_H is identical with unrearranged V_H segment transcripts A1 and A4, which belong to the V_H558 family [Yancopoulos et al., *supra*].

Because the germline genes corresponding to the V_x genes of myeloma MOPC21 V_x19 gene family [Potter et al., *supra*] are unknown, direct evaluation of the somatic mutations of the light chains was not possible. However, O1 and O4 light chain variable regions are identical with the sequence reported for natural autoantibody E7 [Baccala et al., *supra*], and are identical with myeloma MOPC21 V_x segment [Hamlyn et al., *supra*], except for one silent nucleotide change. This provides strong evidence that O1 and O4 V_x segments are directly encoded by germline Ig genes. Though O4 and HNK-1 V had minor differences from germline V_H101 [Katsoka et al, J. Biol. Chem., 257 (1982) 277-285], their sequences are very close to D23 V_H sequence, a well-characterized natural autoantibody [Baccala et al., *supra*]. In addition, HNK-1 V_x showed identity with myeloma MOPC41 [Seidman et al., Nature, 280 (1979), 370-375] and germline V_x41 [Seidman et al., *supra*], except for two silent nucleotide changes. Our results were not able to determine whether A2B5 V_x segment is encoded by germline Ig gene. However, the A2B5 V_x is encoded by an unidentified germline Ig gene

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rather than by extensive somatic mutation of a germline Ig gene.

The results also showed that O1 and O4 react to multiple different antigens as demonstrated by ELISA. This is
5 consistent with the immunocytochemistry [Eisenbarth et al., *supra*; Sommer et al., *supra*] demonstrating the reactivity of these mAbs to intracellular antigens in many cells. HNK-1 was shown previously to be
10 polyreactive by Western blots using the lysates of chick embryo spinal cord neuron-enriched cultures and rat brain [McGarry et al., *supra*].

The Ig cDNA sequences and polyreactivity to multiple antigens are consistent with the hypothesis that O1, O4 and HNK-1 are natural autoantibodies. In contrast, A2B5
15 does not show polyreactivity by ELISA and the Ig cDNA sequence similarity to the germline is undetermined. Characterization of O1, O4 and HNK-1 as natural autoantibodies raises the possibility that they exist normally in serum and have physiologic function during
20 development or in CNS diseases. In support of a physiologic function for these mAbs is the report that O4 stimulates the differentiation of OLs *in vitro* [Bansal et al., *supra*]. Since Schwann cells share with OLs the antigens recognized by O1, O4 and HNK-1, this suggests
25 that these mAbs may have a function not only in the CNS but also in the peripheral nervous system. Direct proof of this hypothesis awaits experiments with these mAbs *in vivo* during development and in animal models of CNS diseases.

30 Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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WHAT IS CLAIMED IS:

- 1 1. A method of stimulating remyelination of central
2 nervous system axons in a mammal in need of such therapy
3 which comprises administer to said mammal an effective
4 amount of a monoclonal autoantibody selected from the
5 group consisting of mAb SCH 94.03, SCH 79.08, O1, O4,
6 A2B5, HNK-1, active fragments thereof, and natural or
7 synthetic autoantibodies having the characteristics
8 thereof.
- 1 2. The method of Claim 1 wherein the method of
2 administration is intravenous administration.
- 1 3. The method of Claim 1 wherein the method of
2 administration is intraperitoneal administration.
- 1 4. The method of Claim 1 wherein said amount of
2 monoclonal antibody administered is between from about
3 0.5 mg/kg to about 400 mg/kg.
- 1 5. A method of stimulating the proliferation of glial
2 cells in central nervous system axons in a mammal in need
3 of such therapy which comprises administering to said
4 mammal an effective amount of a monoclonal autoantibody
5 selected from the group consisting of mAb SCH 94.03, SCH
6 79.08, O1, O4, A2B5, HNK-1, active fragments thereof, and
7 natural or synthetic autoantibodies having the
8 characteristics thereof.
- 1 6. The method of Claim 5 wherein the method of
2 administration is intravenous administration.
- 1 7. The method of Claim 5 wherein the method of
2 administration is intraperitoneal administration.

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1 8. The method of Claim 5 wherein said amount of
2 monoclonal antibody administered is between from about
3 0.5 mg/kg to about 400 mg/kg.

1 9. A method of treating a demyelinating disease of the
2 central nervous system in a mammal in need of such
3 therapy which comprises administering to said mammal an
4 effective amount of a monoclonal autoantibody selected
5 from the group consisting of mAb SCH94.03, SCH79.08, O1,
6 O4, A2B5 and HNK-1, active fragments thereof, and natural
7 or synthetic autoantibodies having the characteristics
8 thereof.

1 10. The method of Claim 9 wherein said mammal is a human
2 being having multiple sclerosis, or a human or domestic
3 animal with a viral demyelinating disease, or a post-
4 neural disease of the central nervous system.

1 11. The method of Claim 9 wherein the method of
2 administration is intravenous administration.

1 12. The method of Claim 9 wherein the method of
2 administration is intraperitoneal administration.

1 13. The method of Claim 9 wherein said amount of
2 monoclonal antibody administered is between from about
3 0.5 mg/kg to about 400 mg/kg.

1 14. The method of Claim 9 wherein said mammal is a mouse
2 infected with Strain DA of Theiler's murine
3 encephalomyelitis virus.

1 15. A *in vitro* method of stimulating the proliferation
2 of glial cells from mixed cell culture comprising:
3 a) culturing a mixed cell culture containing glial
4 cells under condition sufficient for cell proliferation;

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- 5 b) introducing into the mixed culture an effective
6 amount of a monoclonal autoantibody selected from the
7 group consisting of mAb SCH94.03, SCH79.08, O1, O4, A2B5,
8 HNK-1, active fragments thereof, and natural or synthetic
9 autoantibodies having the characteristics thereof,
10 thereby producing a monoclonal antibody-treated mixed
11 culture;
12 c) maintaining the culture of step b) under conditions
13 sufficient for proliferation of monoclonal antibody-
14 treated cells, thereby resulting in the proliferation of
15 glial cells in the mixed culture; and
16 d) harvesting the glial cells from the mixed culture.

1 16. The method of Claim 18 wherein the mixed culture is
2 obtained from rat optic nerve.

1 17. The method of Claim 18 wherein the mixed culture is
2 obtained from rat brain.

3 18. A method of stimulating remyelination of central
4 nervous system axons in a mammal in need of such therapy
5 comprising:

- 6 a) culturing glial cells under conditions sufficient
7 for cell proliferation thereby producing a glial cell
8 culture;
9 b) introducing into the glial cell culture an effective
10 amount of a monoclonal autoantibody selected from the
11 group consisting of mAb SCH94.03, SCH79.08, O1, O4, A2B5,
12 HNK-1, active fragments thereof, and natural or synthetic
13 autoantibodies having the characteristics thereof,
14 thereby producing a monoclonal antibody-treated glial
15 cell culture;
16 c) maintaining the culture of step b) under conditions
17 sufficient for proliferation of monoclonal antibody-
18 treated cells;
19 d) harvesting the monoclonal antibody-treated cells from
20 the culture, thereby obtaining glial cells; and

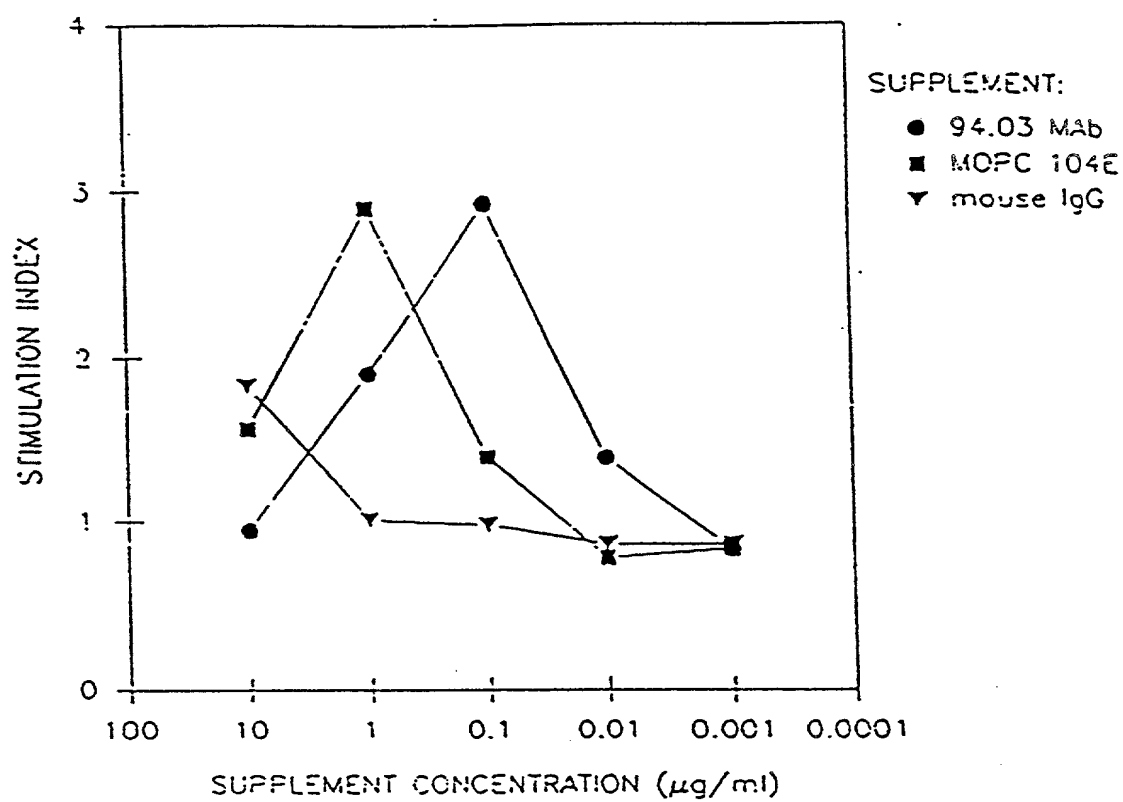
21 e) introducing the glial cells obtained in step d) into
22 the central nervous system of the mammal, thereby
23 stimulating remyelination of central nervous system
24 axons.

1 19. A pharmaceutical composition comprising, as the
2 active agent, an active fragment of a monoclonal
3 autoantibody selected from the group consisting of mAb
4 SCH94.03, SCH79.08, O1, O4, A2B5, HNK-1, and natural or
5 synthetic autoantibodies having the characteristics of
6 mAb SCH94.03, SCH79.08, O1, O4, A2B5 or HNK-1.

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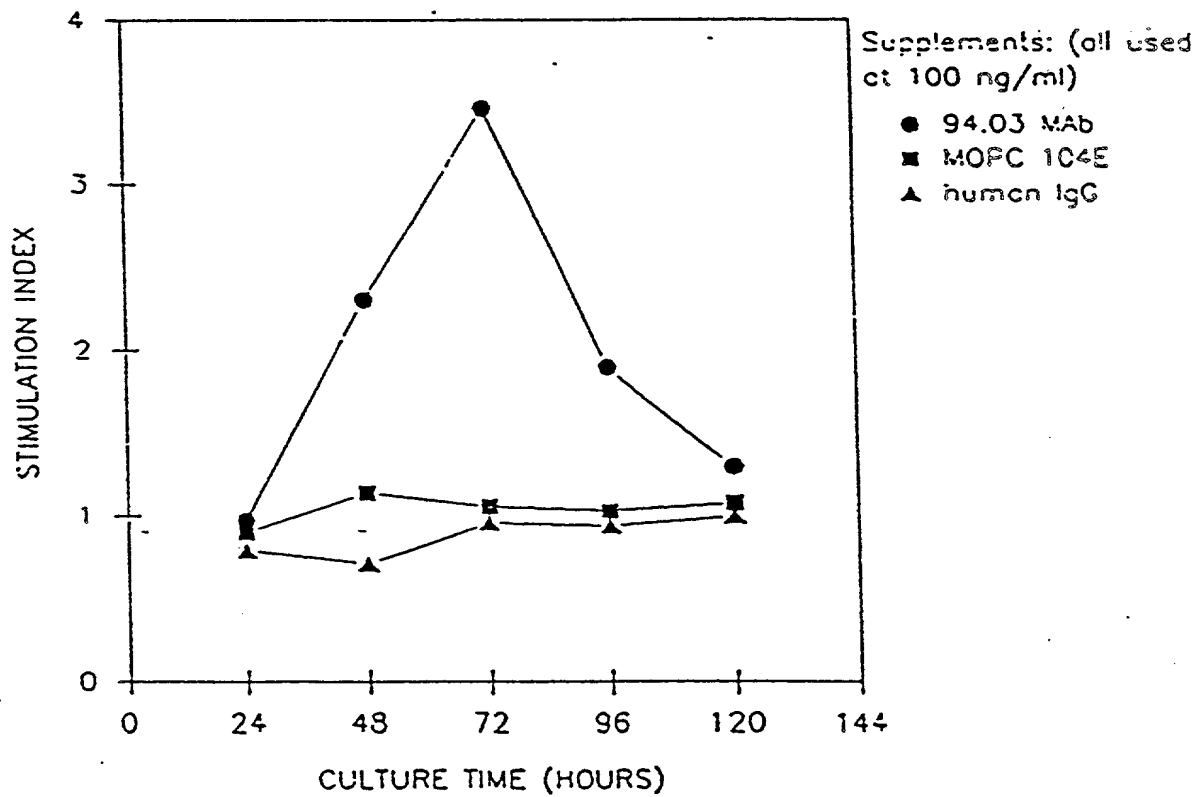
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Figure 1



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Figure 2



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Fig. 3A



Fig. 3B

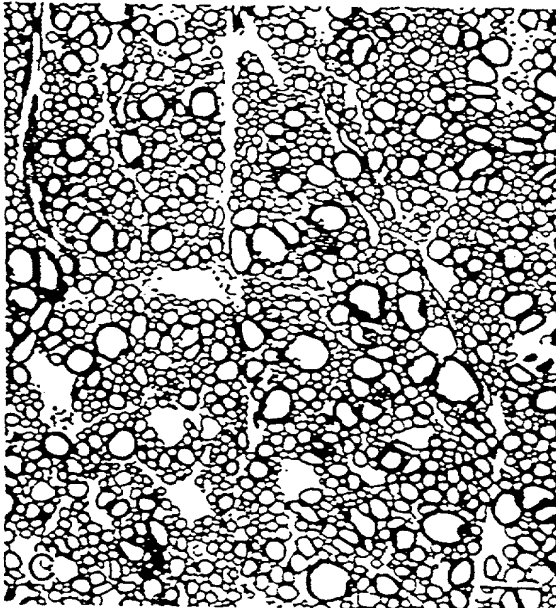
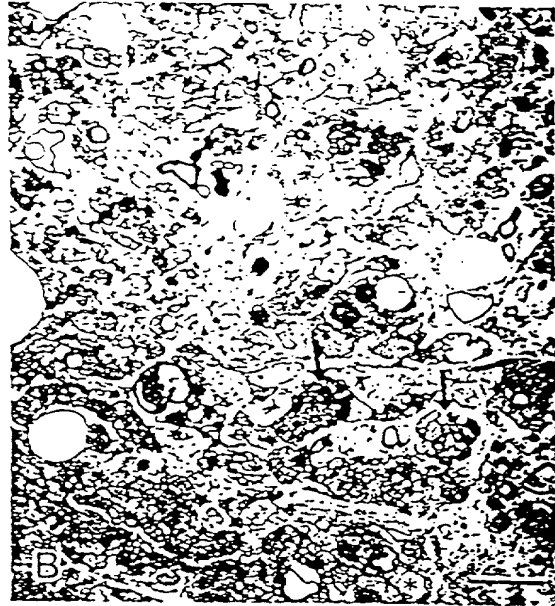


Fig. 3C

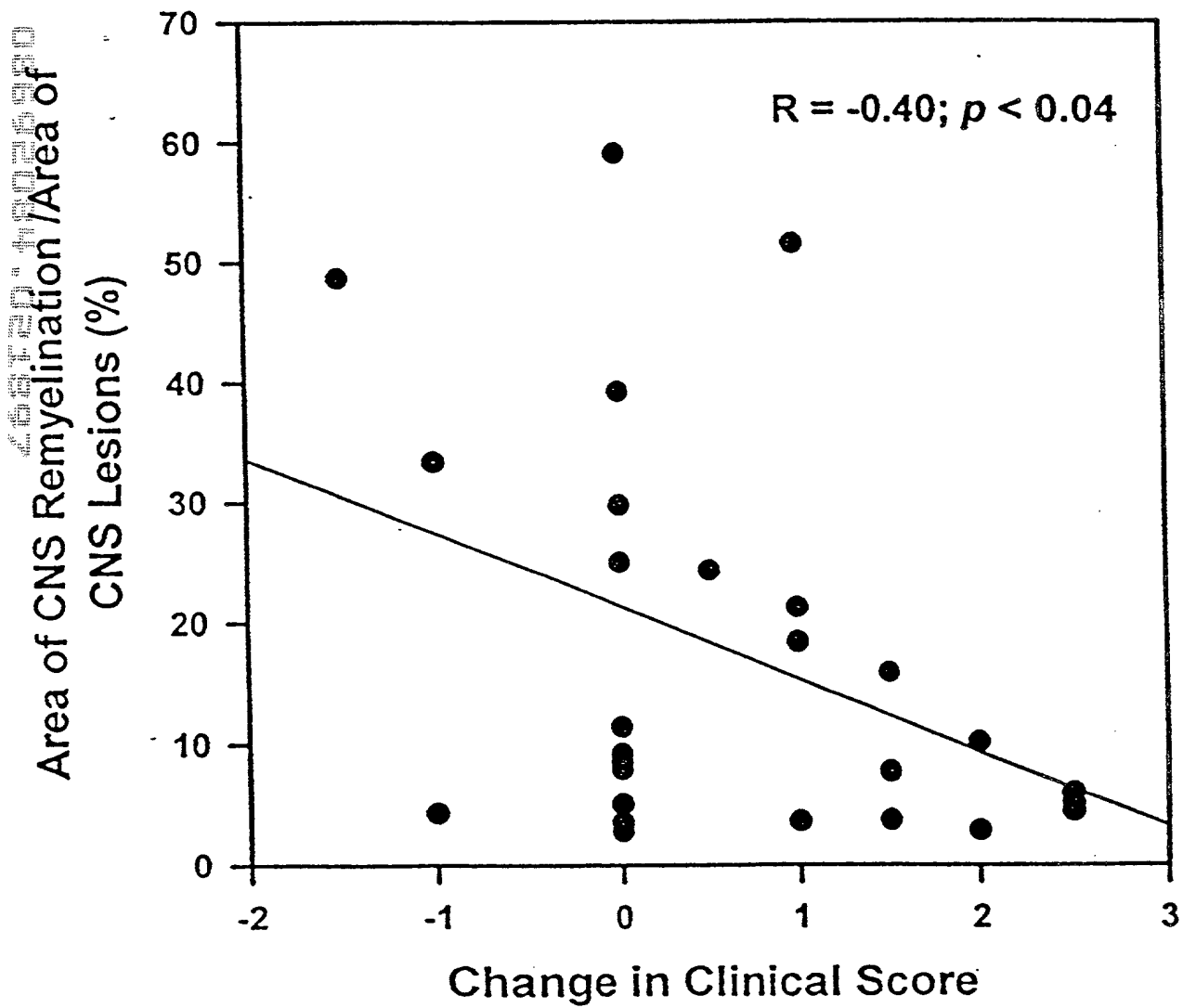


Fig. 3D

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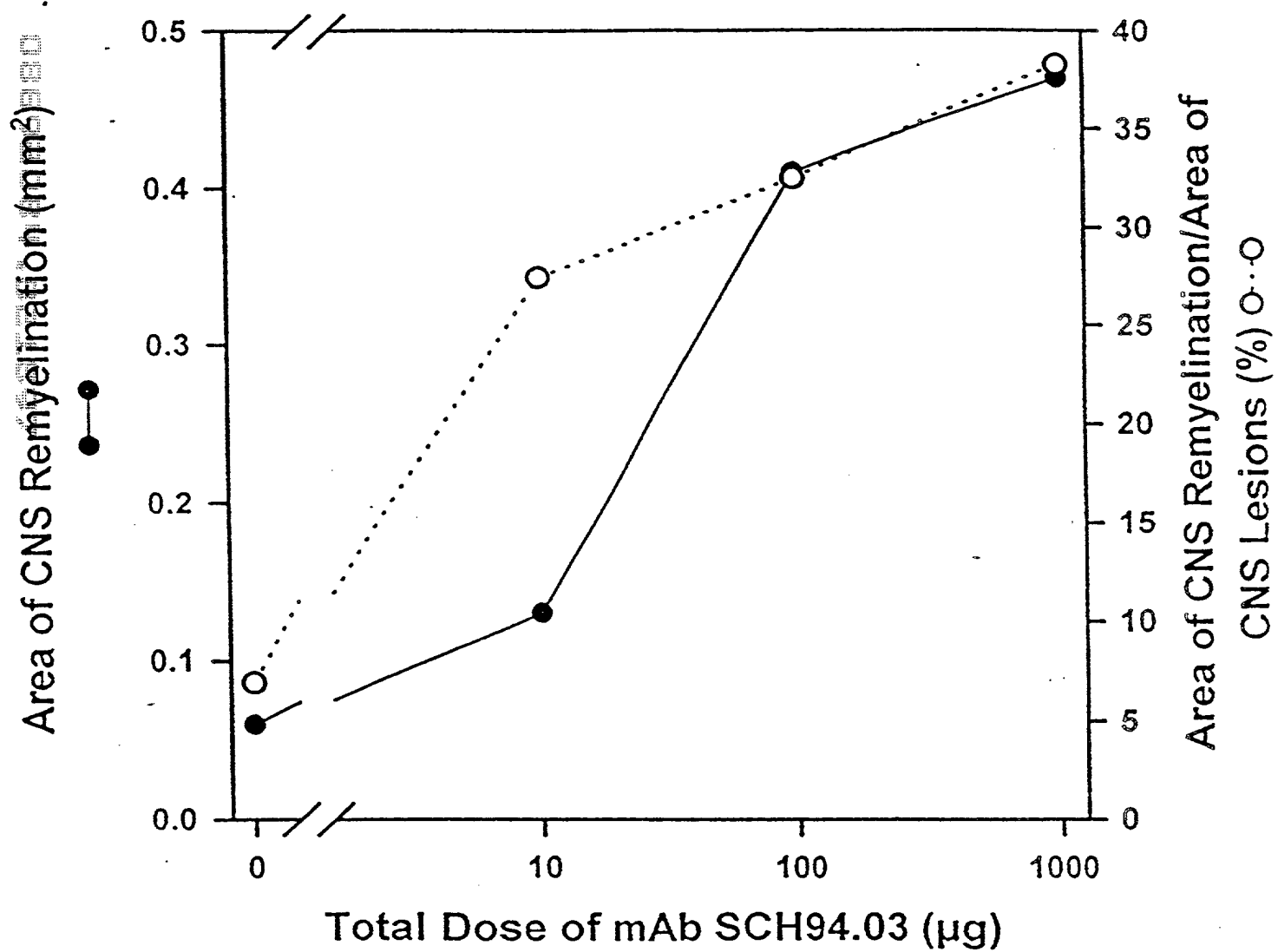
1199-1-001 CIP (Sheet 4 of 18)

Figure 4



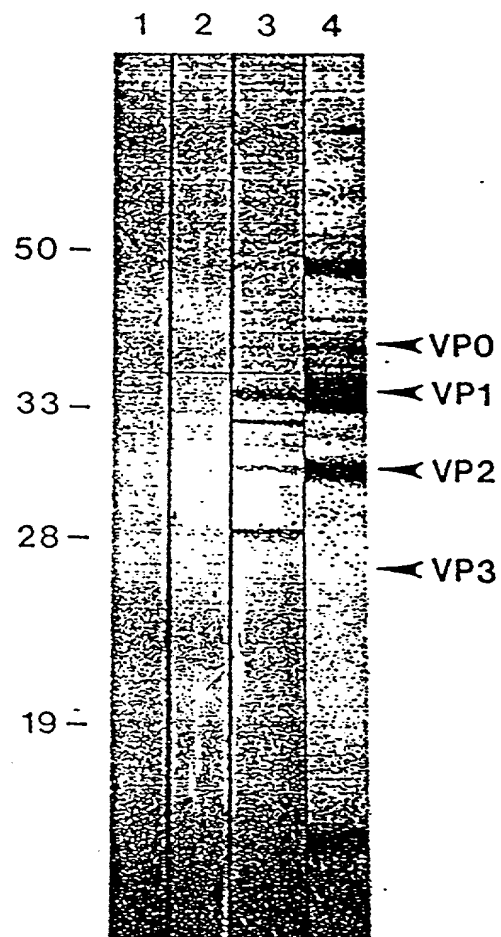
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Figure 5



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Figure 6



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Fig. 7A

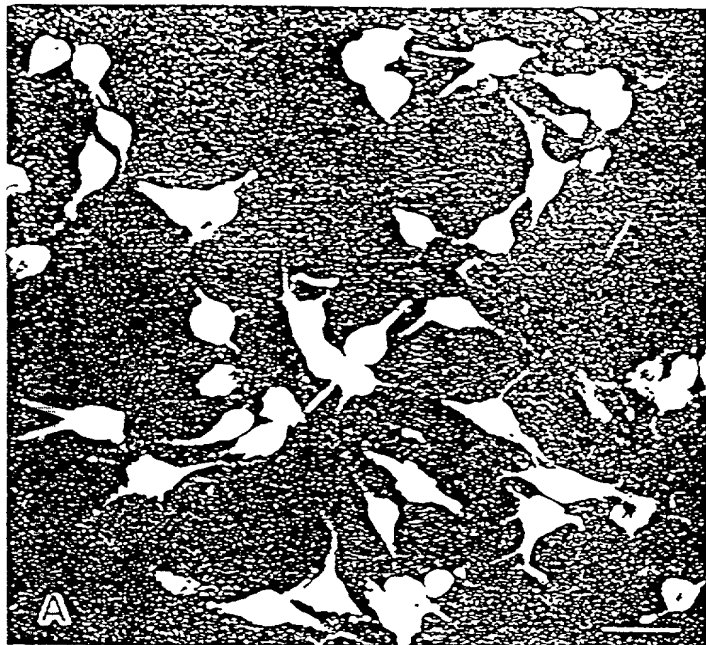


Fig. 7B



Fig. 7C

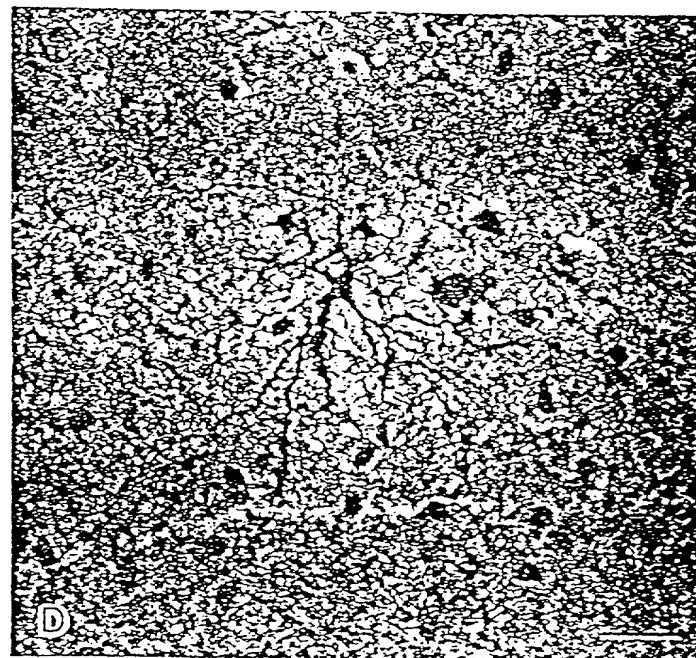


Fig. 7D

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ELISA with SCH94.03 and protein antigens

Fig. 8A

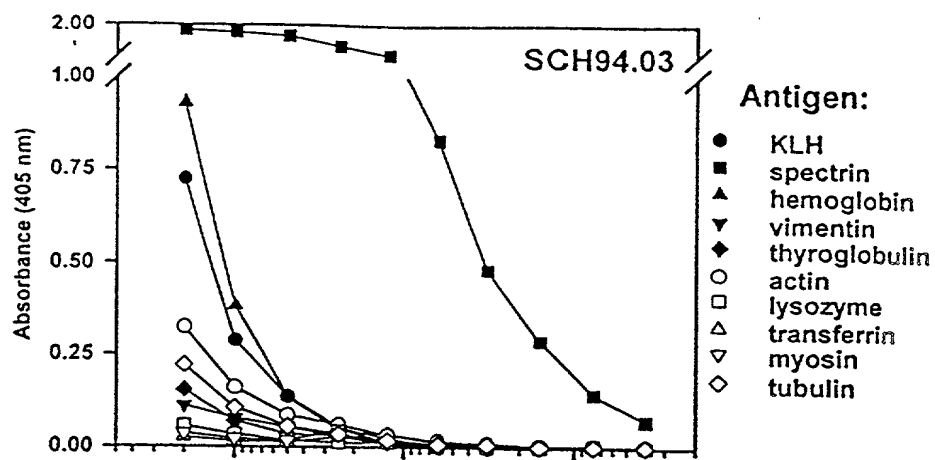


Fig. 8B

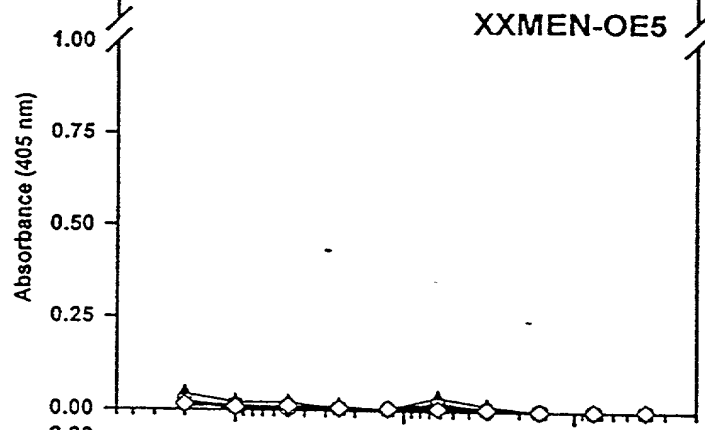


Fig. 8C

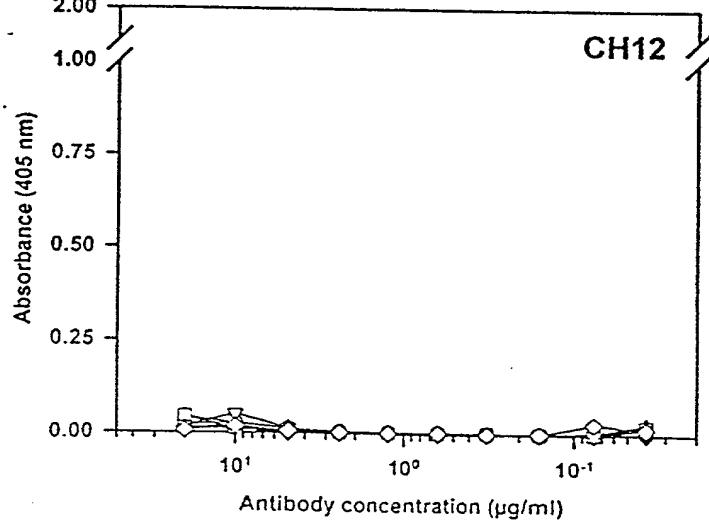
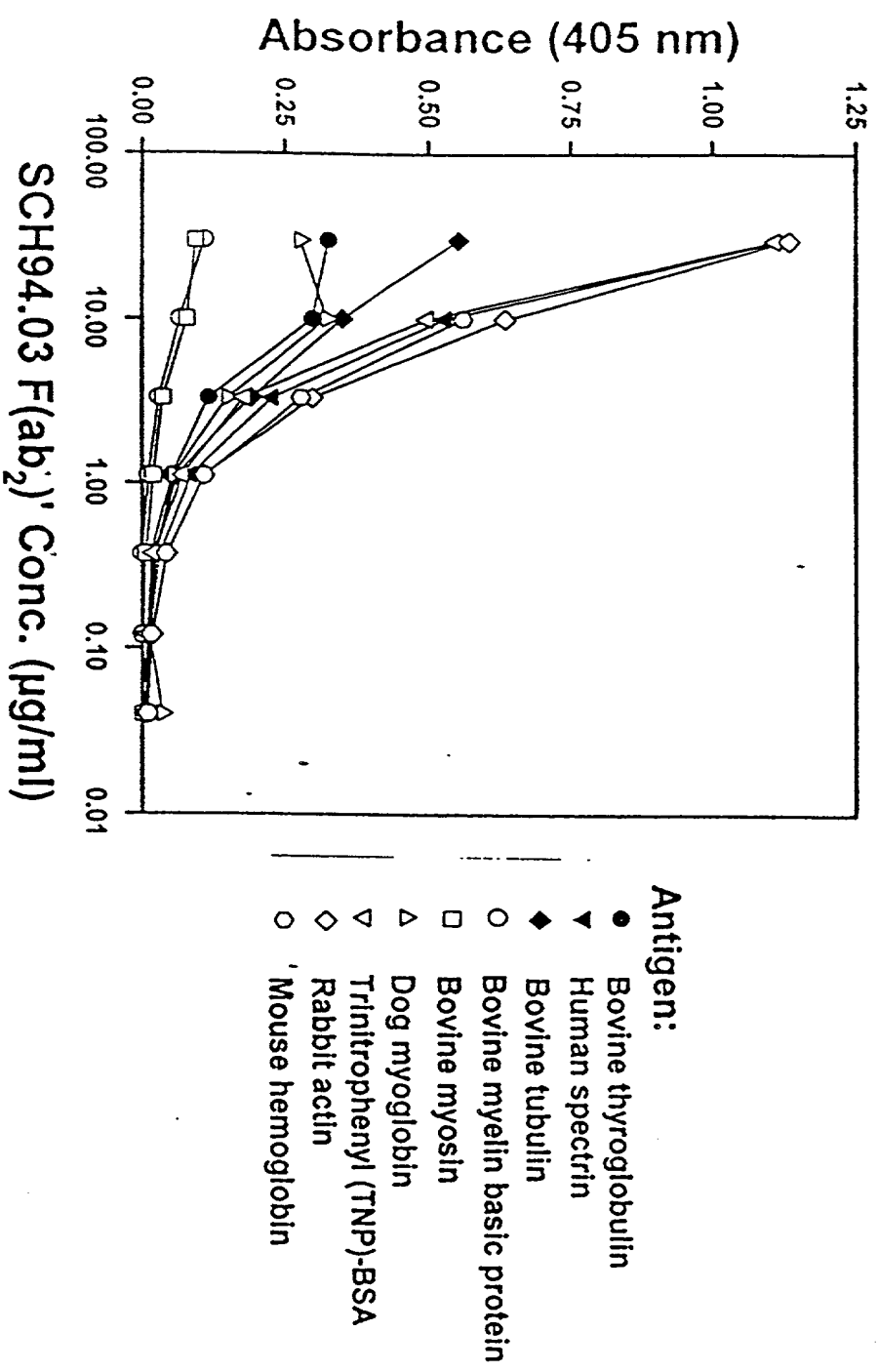


Figure 9 ELISA with SCH94.03 F(ab₂)' fragments



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ELISA with SCH94.03 and chemical haptens

Fig. 10A

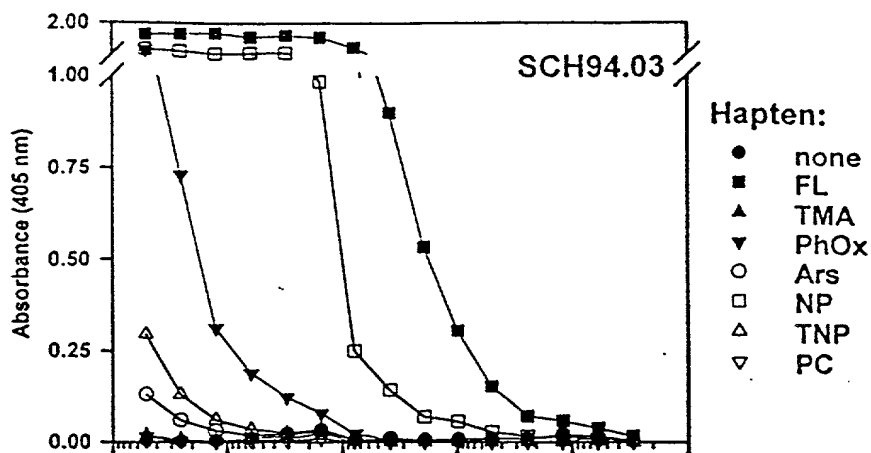


Fig. 10B

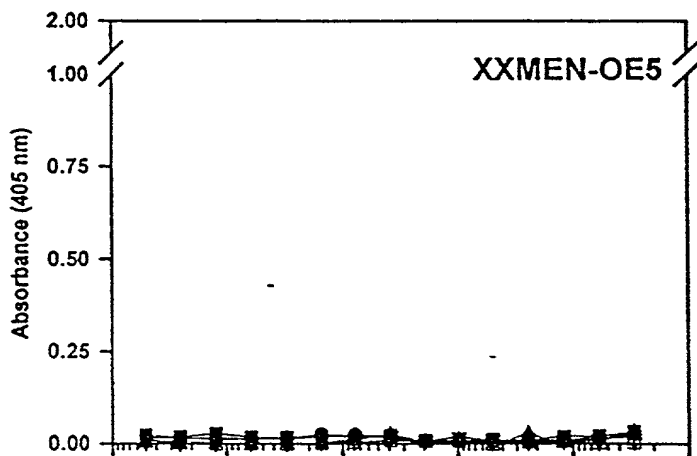
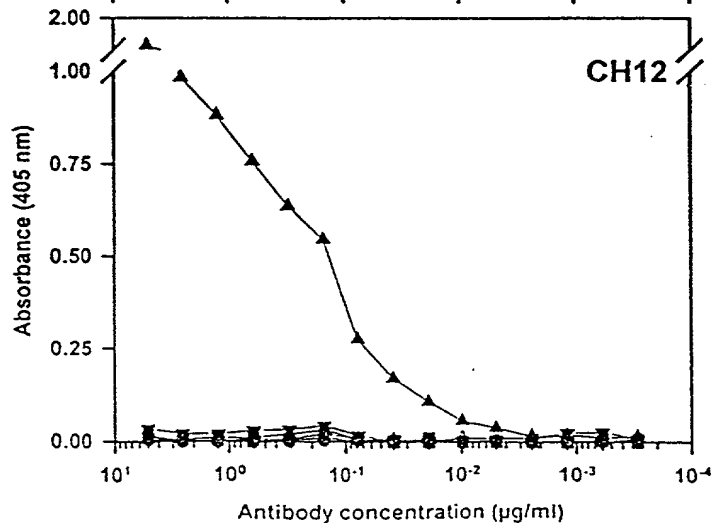


Fig. 10C



GENE4.03
GHI?
germline virus

	-19		-9		1
M N S A Q T L G B L L C P Q Q Y R Q D I					
AND AND TCT CCT CAG TTC CTT CCT CTC CTG TTG CA CCG ACC ACA TGT CAT ATG					

BCN44.03
CM12
germline Y_H?

-17
 A T G C A T G A C G T A T C G T T T G T A C C A C G T A C C C A C G T C M
 A T G C A T G A C G T A T C G T T T G T A C C A C G T A C C C A C G T C M

GEN 4.03
C11?
germano v10

[illegible]

8CM14.03
CM17
germano Vx2

1 0 0 2 0 2 1 1 V K 2 A 2 V K 1 1 0 K A
 C
 C

50000.00
Bill
gemline vrio

[illegible]

6C04.03
CH12
germano V₂O₅

⁰ ¹ ² ³ ⁴ ⁵ ⁶ ⁷ ⁸ ⁹ ¹⁰ ¹¹ ¹² ¹³ ¹⁴ ¹⁵ ¹⁶ ¹⁷ ¹⁸ ¹⁹ ²⁰ ²¹ ²² ²³ ²⁴ ²⁵ ²⁶ ²⁷ ²⁸ ²⁹ ³⁰
 ccc tac acc ttc acc acc tac taa att cgc taa gta aac cgc acc cct cca cca ccc ctt cgc tgg

5044.01
C111
Germano v.10

L I Y T N L K V Q S A
CTG ATC TAC AAC ACA GTG AGA GTA CAG CTC GCA GTC CGA TTA ACC TTC ACT CCG AAT CCG TTC GGA

90 100

. 8C994.03
 CML
 germline Y83

[illegible]

gentle rain

[illegible]

SCB44,03
CBI
germano VZ33

	10										10										
	70	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CTG	ACT	GTA	ACC	AAA	TGC	TGC	ACG	ACA	GGC	TAC	ATG	CGA	CTC	ACG	CTC	ACA	TTC	GAC	GAC	TTC	

2117
 2118
 2119
 2120

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99

CH12
germline VN23
JM2

CCG CTC TAT TAT TGT CCA AAA CGG GCG CG :¹ T² TAC TAC GAT AGC AGC ...³ T⁴ TTT CAC⁵
 ...⁶ ...⁷ ...⁸ ...⁹ ...¹⁰ ...¹¹ ...¹² ...¹³ ...¹⁴ ...¹⁵ ...¹⁶ ...¹⁷ ...¹⁸ ...¹⁹ ...²⁰ ...²¹ ...²² ...²³ ...²⁴ ...²⁵ ...²⁶ ...²⁷ ...²⁸ ...²⁹ ...³⁰ ...³¹ ...³² ...³³ ...³⁴ ...³⁵ ...³⁶ ...³⁷ ...³⁸ ...³⁹ ...⁴⁰ ...⁴¹ ...⁴² ...⁴³ ...⁴⁴ ...⁴⁵ ...⁴⁶ ...⁴⁷ ...⁴⁸ ...⁴⁹ ...⁵⁰ ...⁵¹ ...⁵² ...⁵³ ...⁵⁴ ...⁵⁵ ...⁵⁶ ...⁵⁷ ...⁵⁸ ...⁵⁹ ...⁶⁰ ...⁶¹ ...⁶² ...⁶³ ...⁶⁴ ...⁶⁵ ...⁶⁶ ...⁶⁷ ...⁶⁸ ...⁶⁹ ...⁷⁰ ...⁷¹ ...⁷² ...⁷³ ...⁷⁴ ...⁷⁵ ...⁷⁶ ...⁷⁷ ...⁷⁸ ...⁷⁹ ...⁸⁰ ...⁸¹ ...⁸² ...⁸³ ...⁸⁴ ...⁸⁵ ...⁸⁶ ...⁸⁷ ...⁸⁸ ...⁸⁹ ...⁹⁰ ...⁹¹ ...⁹² ...⁹³ ...⁹⁴ ...⁹⁵ ...⁹⁶ ...⁹⁷ ...⁹⁸ ...⁹⁹ ...¹⁰⁰ ...¹⁰¹ ...¹⁰² ...¹⁰³ ...¹⁰⁴ ...¹⁰⁵ ...¹⁰⁶ ...¹⁰⁷ ...¹⁰⁸ ...¹⁰⁹ ...¹¹⁰ ...¹¹¹ ...¹¹² ...¹¹³ ...¹¹⁴ ...¹¹⁵ ...¹¹⁶ ...¹¹⁷ ...¹¹⁸ ...¹¹⁹ ...¹²⁰ ...¹²¹ ...¹²² ...¹²³ ...¹²⁴ ...¹²⁵ ...¹²⁶ ...¹²⁷ ...¹²⁸ ...¹²⁹ ...¹³⁰ ...¹³¹ ...¹³² ...¹³³ ...¹³⁴ ...¹³⁵ ...¹³⁶ ...¹³⁷ ...¹³⁸ ...¹³⁹ ...¹⁴⁰ ...¹⁴¹ ...¹⁴² ...¹⁴³ ...¹⁴⁴ ...¹⁴⁵ ...¹⁴⁶ ...¹⁴⁷ ...¹⁴⁸ ...¹⁴⁹ ...¹⁵⁰ ...¹⁵¹ ...¹⁵² ...¹⁵³ ...¹⁵⁴ ...¹⁵⁵ ...¹⁵⁶ ...¹⁵⁷ ...¹⁵⁸ ...¹⁵⁹ ...¹⁶⁰ ...¹⁶¹ ...¹⁶² ...¹⁶³ ...¹⁶⁴ ...¹⁶⁵ ...¹⁶⁶ ...¹⁶⁷ ...¹⁶⁸ ...¹⁶⁹ ...¹⁷⁰ ...¹⁷¹ ...¹⁷² ...¹⁷³ ...¹⁷⁴ ...¹⁷⁵ ...¹⁷⁶ ...¹⁷⁷ ...¹⁷⁸ ...¹⁷⁹ ...¹⁸⁰ ...¹⁸¹ ...¹⁸² ...¹⁸³ ...¹⁸⁴ ...¹⁸⁵ ...¹⁸⁶ ...¹⁸⁷ ...¹⁸⁸ ...¹⁸⁹ ...¹⁹⁰ ...¹⁹¹ ...¹⁹² ...¹⁹³ ...¹⁹⁴ ...¹⁹⁵ ...¹⁹⁶ ...¹⁹⁷ ...¹⁹⁸ ...¹⁹⁹ ...²⁰⁰ ...²⁰¹ ...²⁰² ...²⁰³ ...²⁰⁴ ...²⁰⁵ ...²⁰⁶ ...²⁰⁷ ...²⁰⁸ ...²⁰⁹ ...²¹⁰ ...²¹¹ ...²¹² ...²¹³ ...²¹⁴ ...²¹⁵ ...²¹⁶ ...²¹⁷ ...²¹⁸ ...²¹⁹ ...²²⁰ ...²²¹ ...²²² ...²²³ ...²²⁴ ...²²⁵ ...²²⁶ ...²²⁷ ...²²⁸ ...²²⁹ ...²³⁰ ...²³¹ ...²³² ...²³³ ...²³⁴ ...²³⁵ ...²³⁶ ...²³⁷ ...²³⁸ ...²³⁹ ...²⁴⁰ ...²⁴¹ ...²⁴² ...²⁴³ ...²⁴⁴ ...²⁴⁵ ...²⁴⁶ ...²⁴⁷ ...²⁴⁸ ...²⁴⁹ ...²⁵⁰ ...²⁵¹ ...²⁵² ...²⁵³ ...²⁵⁴ ...²⁵⁵ ...²⁵⁶ ...²⁵⁷ ...²⁵⁸ ...²⁵⁹ ...²⁶⁰ ...²⁶¹ ...²⁶² ...²⁶³ ...²⁶⁴ ...²⁶⁵ ...²⁶⁶ ...²⁶⁷ ...²⁶⁸ ...²⁶⁹ ...²⁷⁰ ...²⁷¹ ...²⁷² ...²⁷³ ...²⁷⁴ ...²⁷⁵ ...²⁷⁶ ...²⁷⁷ ...²⁷⁸ ...²⁷⁹ ...²⁸⁰ ...²⁸¹ ...²⁸² ...²⁸³ ...²⁸⁴ ...²⁸⁵ ...²⁸⁶ ...²⁸⁷ ...²⁸⁸ ...²⁸⁹ ...²⁹⁰ ...²⁹¹ ...²⁹² ...²⁹³ ...²⁹⁴ ...²⁹⁵ ...²⁹⁶ ...²⁹⁷ ...²⁹⁸ ...²⁹⁹ ...³⁰⁰ ...³⁰¹ ...³⁰² ...³⁰³ ...³⁰⁴ ...³⁰⁵ ...³⁰⁶ ...³⁰⁷ ...³⁰⁸ ...³⁰⁹ ...³¹⁰ ...³¹¹ ...³¹² ...³¹³ ...³¹⁴ ...³¹⁵ ...³¹⁶ ...³¹⁷ ...³¹⁸ ...³¹⁹ ...³²⁰ ...³²¹ ...³²² ...³²³ ...³²⁴ ...³²⁵ ...³²⁶ ...³²⁷ ...³²⁸ ...³²⁹ ...³³⁰ ...³³¹ ...³³² ...³³³ ...³³⁴ ...³³⁵ ...³³⁶ ...³³⁷ ...³³⁸ ...³³⁹ ...³⁴⁰ ...³⁴¹ ...³⁴² ...³⁴³ ...³⁴⁴ ...³⁴⁵ ...³⁴⁶ ...³⁴⁷ ...³⁴⁸ ...³⁴⁹ ...³⁵⁰ ...³⁵¹ ...³⁵² ...³⁵³ ...³⁵⁴ ...³⁵⁵ ...³⁵⁶ ...³⁵⁷ ...³⁵⁸ ...³⁵⁹ ...³⁶⁰ ...³⁶¹ ...³⁶² ...³⁶³ ...³⁶⁴ ...³⁶⁵ ...³⁶⁶ ...³⁶⁷ ...³⁶⁸ ...³⁶⁹ ...³⁷⁰ ...³⁷¹ ...³⁷² ...³⁷³ ...³⁷⁴ ...³⁷⁵ ...³⁷⁶ ...³⁷⁷ ...³⁷⁸ ...³⁷⁹ ...³⁸⁰ ...³⁸¹ ...³⁸² ...³⁸³ ...³⁸⁴ ...³⁸⁵ ...³⁸⁶ ...³⁸⁷ ...³⁸⁸ ...³⁸⁹ ...³⁹⁰ ...³⁹¹ ...³⁹² ...³⁹³ ...³⁹⁴ ...³⁹⁵ ...³⁹⁶ ...³⁹⁷ ...³⁹⁸ ...³⁹⁹ ...⁴⁰⁰ ...⁴⁰¹ ...⁴⁰² ...⁴⁰³ ...⁴⁰⁴ ...⁴⁰⁵ ...⁴⁰⁶ ...⁴⁰⁷ ...⁴⁰⁸ ...⁴⁰⁹ ...⁴¹⁰ ...⁴¹¹ ...⁴¹² ...⁴¹³ ...⁴¹⁴ ...⁴¹⁵ ...⁴¹⁶ ...⁴¹

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Figure 12

Leader Peptide	
AI/A4 OI	<p>-19 M G W R W I F L F L L S G T A G V H C +1 ATG GGA TGG AGA TGG ATC TTT CTT TTC CTC CTG TCA GGA ACT GCA GGT GTC CAT TGC CAG GTT CAG CTG CAG CAG TCT GGA CCT</p>
AI/A4 OI	<p>10 E L V K P G A L V K I S C K A S G Y T F T A C C TTC A C Y D I N W V GAG CTG GTG AAG CCT GGG GCT TTA GTG AAG ATA TCC TGC AAG GCT TCT GGT TAC ACC TTC ACA AGC TAC GAT ATA AAC TGG GTG</p>
AI/A4 OI	<p>40 K Q R P G Q G L E W I G W I Y P P G D G S T K Y N E K F K AAG CAG AAG CCT GGA CAG GGA CTT GAG TGG ATT GGA TGG ATT TAT CCT GGA GAT GGT AGT ACT AAG TAC AAT GAG AAA TTC AAG</p>
AI/A4 OI	<p>70 G K A T L T A A D K S S S T A Y M Q L S 82A 82B 82C GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC AGC AGC CTG ACT TCT GAG AAC TCT GCA GTG</p>
AI/A4 OI	<p>90 Y F C A R G A R F Y W Y F D V W G A G T T V T V S S TAT TTC TGT GCA AGA GGG GCC AGG TTC TAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG ACG GTC ACC GTT TCC TCA GAG AGT</p>
AI/A4 gemline JH1 OI	<p>100 100A 100B TAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG ACG GTC ACC GTT TCC TCA GAG AGT</p>

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Leader Peptide

-19
M A V L G L L F C L V T F P S C V L S +1
ATG GCT GTC TTA GGG CTG CTC TTC TCC CTA GTG ACA TTC CCA AGC GGT GTC CTA TCC CAG GTG CAG CTG AAG CAG TCA GGA CCT
gemline VH101
O4
HNK-1
D23

10
G L V Q P S Q S L S I T C T V S G F S L T T S Y G V H W V
GGC CTA GTG CAG CCC TCA CAG AGC CTG TCC ATC ACC TCC ACA GTC TCT GGT TTC TCA TTA ACT AGC TAT GGT GTA CAC TGG GTT
gemline VH101
O4
HNK-1
D23

CDR2

40
R Q S P G K G Q L E W L G V I W S G G G S T D Y N A A F I S
CCG CAG TCT CCA GGA AAG GGT CTG GAG TGG CTG GGA GTG ATG TGG AGT GGT GGA AGC ACA GAC TAT AAT GCA GCT TTC ATA TCC
gemline VH101
O4
HNK-1
D23

CDR3

J region

100 100A 100B 100C 100D
Y Y A M D Y W G Q G T S V T V S S
TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
gemline VH101
gemline J14
O4
HNK-1
D23

CDR3

100
D C G S R G
GAC TAC GGT AGT AGG GGG
HNK-1
D23

[illegible]

		Leader Peptide																											
gemline VI A2B5	-19 M ATG	K	L	W	L	N	W	V	F	L	L	T	L	L	L	H	G	I	Q	C	+1 E	V	K	L	V	E	S	G	G
		AAO	TTO	TGG	TTA	AAO	TGG	GTT	TTT	CTT	TTA	ACA	CTT	TTA	CAT	GGT	ATC	CAO	TGT	GAG	GTO	AAO	CTO	GTO	GAA	TCT	GGA	GGA	T
gemline VI A2B5	10 G GGC	L	V	Q	P	G	G	S	L	R	L	S	C	A	T	S	G	F	T	F	S	D	F	Y	M	B	W	V	
		CTT	GTA	CAO	CCT	GGG	GGT	TCT	CTG	AGA	CTC	TCC	TGT	GCA	ACT	TCT	GGG	TTC	ACC	TTC	AGT	GAT	TTC	TAC	ATG	GAO	TGG	GTC	
gemline VI A2B5	40 R CCC	Q	P	P	G	K	R	L	B	W	I	A	A	S	R	N	K	A	N	D	Y	T	T	B	Y	S	A	S	
		CAO	CCT	CCA	GGG	AAO	AGA	CTG	GAO	TGG	ATT	GCT	GCA	AGT	AGA	AAO	AAA	GCT	AAO	GAT	TAT	ACA	ACA	GAO	TAC	AGT	GCA	TCT	
gemline VI A2B5	70 V GTG	K	G	R	F	I	V	S	R	D	T	S	Q	S	I	L	Y	L	Q	M	N	A	L	R	A	B	D	T	
		AAO	GGT	COG	TTC	ATC	GTC	TCC	AGA	GAC	ACT	TCC	CAA	AGC	ATC	CTC	TAC	CTT	CAO	ATG	AAO	GCC	CTG	AGA	GCT	GAO	GAC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	90 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
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gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q	G	T	L	V	T	
		ATT	TAT	TAC	TGT	GCA	AGA	GAT	GCA	C						GCC	TGG	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	
gemline VI A2B5	100 A GCC	I	Y	Y	C	A	R	D	A	R	Q	L	G	L	P	A	W	F	A	Y	W	G	Q						

[illegible]

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Figure 16

Leader Peptide	
gemline Vκ41 HNK-1 MOPC41	M D M R A P A Q I F G F L L L L L F Q G T R C D I Q M T Q ATG GAC ATG AGG GCT CCT GCA CAG ATT TTT GGC TTC TTG TTG CTC TTG TTT CAA GGT ACC AGA TGT GAC ATC CAG ATG ACC CAG
gemline Vκ41 HNK-1 MOPC41	S P S S L S A S L G H R V S L T C R A S Q D I G S S L N TCT CCA TCC TCC TTA TCT GCC TCT CTG GGA GAA AGA GTC AGT CTC ACT TGT CGG GCA AGT CAG GAC ATT GGT AGT AGC TTA AAC
gemline Vκ41 HNK-1 MOPC41	W L Q Q E P D G T I K R L I Y A T S S L D S G V P K R F TGG CTT CAG CAG GAA CCA GAT GGA ACT ATT AAA CGC CTG ATC TAC GCC ACA TCC AGT TTA GAT TCT GGT GTC CCC AAA AGG TTC
gemline Vκ41 HNK-1 MOPC41	S G S R S G S D Y S L T I S S L E S E D F V D Y Y C L Q AGT GGC AGT AGG TCT GGG TCA GAT TAT TCT CTC ACC ATC AGC AGC CTT GAG TCT GAA GAT TTT GTA GAC TAT TAC TGT CTA CAA
gemline Vκ41 HNK-1 MOPC41	Y A S S P Y T F G G T K L E I K R TAT GCT AGT TCT CCT TAC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT TCA

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[illegible][illegible]

I 199-1-001 CIP (Sheet 18 of 18)

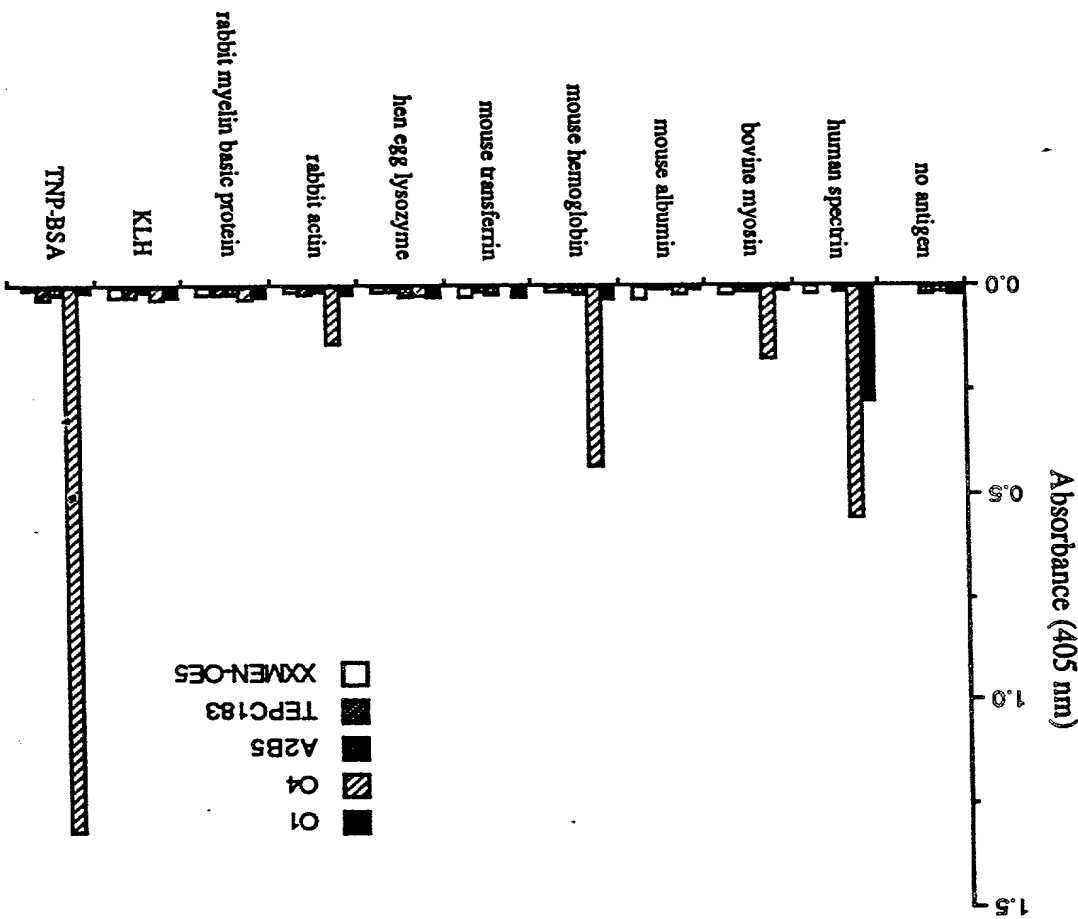


Figure 18



02/18/97

Patent
Attorney's Docket No. 1199-1-001 CIP

#3

Applicant or Patentee: Moses Rodriguez; David J. Miller; and Kunihiro AsakuraApplication or Patent No.: 08/692,084Filed or Issued: August 8, 1996For: MAYO FOUNDATION FOR MEDICAL RESEARCH**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Acorda TherapeuticsADDRESS OF CONCERN 145 West 58th Street, Apt. 8JNew York, New York 10119

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.12, and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average, over the previous fiscal year of the concern, of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES by inventor(s) Moses Rodriguez; David J. Miller; and Kunihiro Asakura described in

- ☐ the specification filed herewith
☒ Application No. 08/692,084, filed August 8, 1996
☐ Patent No. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern, or organization having rights to the invention is listed below,* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27.)

253720 4802530

NAME MAYO FOUNDATION FOR MEDICAL RESEARCH

ADDRESS 200 First Street, SW, Rochester, Minnesota 55905

☐ individual ☐ small business concern ☒ nonprofit organization

NAME _____

ADDRESS _____

☐ individual ☐ small business concern ☐ nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee and any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b).)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Ron Cohen, M.D.

TITLE OF PERSON OTHER THAN OWNER President

ADDRESS OF PERSON SIGNING 145 West 58th Street, Apt. 8J
New York, New York 10119

SIGNATURE _____

DATE 11/1/96



02/18/97

Patent
Attorney's Docket No. 1199-1-001 CIPApplicant or Patentee: Moses Rodriguez; David J. Miller; and Kunihiko AsakuraApplication or Patent No.: 08/692,084Filed or Issued: August 8, 1996For: PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) AND 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION MAYO FOUNDATION FOR MEDICAL RESEARCHADDRESS OF ORGANIZATION 200 First Street, SW, Rochester, Minnesota 55905

TYPE OF ORGANIZATION

- ☐ University or other institution of higher education
- ☐ Tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3))
- ☒ Nonprofit scientific or educational under statute of state of The United States of America
(Name of state _____)
(Citation of statute _____)
- ☐ Would qualify as tax exempt under Internal Revenue Service Code (26 U.S.C. §§ 501(a) and 501(c)(3)) if located in The United States of America
- ☐ Would qualify as nonprofit scientific or educational under statute of The United States of America if located in The United States of America
(Name of state _____)
(Citation of statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, with regard to the invention entitled PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES by inventor(s) Moses Rodriguez; David J. Miller; and Kunihiko Asakura described in

- ☐ the specification filed herewith
- ☒ Application No. 08/692,084, filed August 8, 1996
- ☐ Patent No. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern, or organization having rights to the invention is listed below,* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an individual inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27.)

FULL NAME ACORDA THERAPEUTICS

ADDRESS 145 West 58th Street, Apt. 8J, New York, New York 10019

☐ individual ☒ small business concern ☐ nonprofit organization

FULL NAME _____

ADDRESS _____

☐ individual ☐ small business concern ☐ nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee and any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b).)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Rick F. Colvin

TITLE IN ORGANIZATION Assistant Treasurer

ADDRESS OF PERSON SIGNING 200 First Street SW, Rochester, Minnesota 55905

SIGNATURE Rick F. Colvin DATE 11/1/96



02/18/97

Attorney Docket No.: 1199-1-001 CIP

#3

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under my name.

We believe that we are the original, first and sole inventors (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES

the Specification of which

☐ is attached hereto
☒ was filed on August 8, 1996
 as Application Serial No. 08/692,084
 and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u>	<u>PRIOR FILED APPLICATION(S)</u>	<u>PRIORITY</u>
<u>NUMBER</u>	<u>COUNTRY (DAY/MONTH/YEAR FILED)</u>	<u>CLAIMED</u>

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08692084, 024597

APPLICATION NO. _____	FILING DATE (DAY/MONTH/YEAR) _____	STATUS - PATENTED, PENDING, ABANDONED _____
08/236,520	29/April/1994	PENDING

We hereby appoint as my attorneys or agents the following persons: Jack Matalon, (Attorney, Registration No. 22,241); Stefan J. Klauber, (Attorney, Registration No. 22,604); David A. Jackson, (Attorney, Registration No. 26,742); Barbara L. Renda, (Attorney, Registration No. 27,626); Paul F. Fehlner, (Attorney, Registration No. 35,135); Joseph M. Homa, (Attorney, Registration No. 40,023) and Michael D. Davis, (Attorney, Registration No. 39, 161) said attorneys or agents to have full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

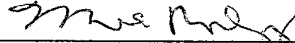
Please address all correspondence regarding this application to:

DAVID A. JACKSON, ESQ.
KLAUBER & JACKSON
411 HACKENSACK AVENUE
HACKENSACK, NEW JERSEY 07601

Direct all telephone calls to David A. Jackson at (201) 487-5800.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR: ¹⁰⁰ MOSES RODRIGUEZ
RESIDENCE: Rochester, Minnesota MN
COUNTRY OF CITIZENSHIP: United States of America
POST OFFICE ADDRESS: 2402 Hillside Lane S.W.
Rochester, Minnesota 55902

SIGNATURE OF INVENTOR 

DATE 2/12/97

FULL NAME OF SECOND INVENTOR: DAVID J. MILLER
RESIDENCE: Fallston, Maryland
COUNTRY OF CITIZENSHIP: United States of America
POST OFFICE ADDRESS: 1501 Fallston Road
Fallston, Maryland 21047

SIGNATURE OF INVENTOR _____

DATE _____

300
FULL NAME OF THIRD INVENTOR: KUNIHICO ASAKURA
RESIDENCE: Rochester, Minnesota
COUNTRY OF CITIZENSHIP: Japan
POST OFFICE ADDRESS: 101 Civic Center Drive N.E.
Room 110
Rochester, Minnesota 55906

SIGNATURE OF INVENTOR ~~David J. Miller~~ Kunihiko Asakura

DATE 2/12/97



02/18/97

Attorney Docket No.: 1199-1-001 CIP #3

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under my name.

We believe that we are the original, first and sole inventors (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES

the Specification of which

- ☐ is attached hereto
☒ was filed on August 8, 1996
 as Application Serial No. 08/692,084
 and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u>	<u>PRIOR FILED APPLICATION(S)</u>	<u>PRIORITY</u>
<u>NUMBER</u>	<u>COUNTRY (DAY/MONTH/YEAR FILED)</u>	<u>CLAIMED</u>

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION NO. _____	FILING DATE (DAY/MONTH/YEAR) _____	STATUS - PATENTED, PENDING, ABANDONED _____
08/236,520	29/April/1994	PENDING

We hereby appoint as my attorneys or agents the following persons: Jack Matalon, (Attorney, Registration No. 22,241); Stefan J. Klauber, (Attorney, Registration No. 22,604); David A. Jackson, (Attorney, Registration No. 26,742); Barbara L. Renda, (Attorney, Registration No. 27,626); Paul F. Fehlner, (Attorney, Registration No. 35,135); Joseph M. Homa, (Attorney, Registration No. 40,023) and Michael D. Davis, (Attorney, Registration No. 39, 161) said attorneys or agents to have full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

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Direct all telephone calls to David A. Jackson at (201) 487-5800.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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